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## REMARKS

In view of the above amendments and the following remarks reconsideration of the outstanding office action is respectfully requested. Support for addition of the “being present in somatic cells of the mammal” limitation in claims 67 and 73 is found on page 1, lines 17-18 of the specification which states the subject recombinatorial substrate can be activated to effect a gain or loss of function of genes in somatic and/or germ cells of a mammal.

The March 22, 2004, personal interview between Examiner Crouch, inventor Howard Federoff, M.D., Ph.D., and applicant’s undersigned attorney is gratefully acknowledged. The substance of that interview is summarized below.

The rejection of claims 67-76 under 35 U.S.C. § 112 (1<sup>st</sup> para.) for lack of enablement is respectfully traversed.

As set forth, in the Amendment under 37 C.F.R. § 1.116, filed on September 26, 2003, applicants submit that one of ordinary skill in the art would have been fully able to practice the present invention by making transgenic mammals from embryonic stem cells which were available for a variety of species at the time of the present invention.

In any event, even if this were not true (which is not the case), one of ordinary skill in the art would recognize that the present invention can be carried out on chimeric (i.e. pluripotent) mammals; totipotency is not required. Indeed, as noted above, page 1, lines 17-18 of the present application teaches that the recombinatorial substrate of the present invention can be activated to effect a gain or loss of function of genes in somatic and/or germ cells of a mammal. Applicant believes that only a fraction of the cells in an organism need be transformed in order to treat a condition.

The accompanying Declaration of Howard Federoff M.D., Ph.D under 37 C.F.R. § 1.132 (“Federoff Declaration”) is submitted to demonstrate that skilled scientists working in the field would recognize that the present invention could be used in conjunction with transgenic mammals and chimeric mammals for therapeutic benefit (Federoff Declaration ¶ 4).

The term “transgenic” refers to the presence of a foreign gene in a cellular context where it can be carried either as an integrated DNA segment or as an episome (Federoff Declaration ¶ 5). Cells that harbor a foreign piece of DNA, therefore, can be considered to be transgenic (Id.).

In the context of mammals, the most common usage of the term “transgenic” pertains to mammals that carry stably integrated copies of a gene of interest and which are conveyed to their progeny through the germ line (Federoff Declaration ¶ 6). During the construction of conventional transgenic animals, the male pronucleus of a fertilized egg is injected with DNA (Id.). That DNA has the opportunity during the next several cell divisions to undergo integration into one or several chromosomal locations (Id.). After transfer of that injected egg into a pseudo pregnant mother, offspring are born that carry the integrated transgene (Id.). The transgene is often found in most, if not all, of the cells including the germ line of the founder animal (Id.). Founders need not carry transgenes in every cell and, therefore, would be considered on that basis to be chimeric (Id.).

Chimeric mammals, carrying a recombinatorial substrate in a subset of cells, would for the purposes of the present invention be suitable substrates for the delivery of cre recombinase (Federoff Declaration ¶ 7). If the transgene in the chimeric founder animal is a recombinatorial substrate, it could support cre recombinase-mediated recombination and, following successful recombination, would express only in cells and tissues capable of recognizing the promoter driving its expression (Id.). The more frequent usage of chimeras refer to those derived from the construction of mammals where an embryonic stem (ES) cell is typically engineered by homologous recombination to harbor a loss or conditional loss of function mutation (Id.). These cells are then used to produce a transgenic mammal by injection of genetically altered ES cells into blastocysts (Id.). When these embryos are transferred to a pseudo pregnant mother, they give rise to chimeras (Id.). As in the pronuclear injection example, not every cell will harbor the transgene (Id.). Typically if those cells that harbor the transgene include the germline, there is an opportunity for it to be conveyed to the progeny of the mammal (Id.).

With regard to the present invention, no distinction need be made between a transgenic mammal in which the transgene is found in all cells versus those mammals where the transgene might be found in a more circumscribed set of cells (Federoff Declaration ¶ 8). To practice the present invention, cre recombinase can be delivered to either a founder mammal derived from pronuclear injection or a mammal derived from the introduction of genetically manipulated ES cells into the blastocyst (Id.). Thus, the present invention can be practiced independently of the source or nature of the transgene in the mammal in which the transgene is carried (Id.).

The present invention has potential human therapeutic importance and was indeed a driving force for its creation (Federoff Declaration ¶ 9). Unfortunate individuals with inborn errors of metabolism (e.g., Mucopolysaccharidoses, Canavan's Disease, Hurler's Disease, Tay-Sachs, among many others) and those with later-onset recessive mutations producing devastating clinical complications (e.g., Juvenile Batten's Disease, etc.) would be suitable recipients for therapy based on the present invention (Id.). This group of disorders share the characteristic that the replacement of the missing and mutated enzyme can be accomplished in a subset of cells and that these gene-modified cells provide sufficient enzymatic function through 'cross-correction' to ameliorate the clinical phenotype (Id.). Such cross-correction can be achieved by administration of gene-modified bone marrow-derived cells (Id.).

However, this same enzymatic correction could be accomplished by other somatic cells genetically engineered to provide the missing enzyme (Federoff Declaration ¶ 10). This principle underlies the therapeutic practice of the present invention (Id.). For example, an individual diagnosed with one of these disorders would have a somatic cell population engineered to carry a recombinatorial substrate that would include an inactive form of the missing enzyme (Id.). The cells to be modified with the recombinatorial substrate could be bone marrow-derived, precursors or skeletal muscle cells, as non-limiting examples for discussion purposes (Id.). Depending on the particular disease, it will be necessary to titrate the amount of replacement enzyme made available to effectively reverse the clinical phenotype (Id.). This would be achieved by the introduction of cre recombinase to a fraction of the cell type(s) harboring the recombinatorial substrate and should result in the expression of the missing enzyme (Id.). The ability to titrate recombination will allow for precise control of the amount of enzyme that is to be replaced (Id.). Given that in some cases the gene-corrected cells, produced by cre-mediated recombination, can migrate and marginate (e.g., bone marrow derived macrophages can enter the brain and other organs), it is likely that wide-spread correction of the clinical response would be observed (Id.).

In summary, transgenic mammals are those that have a transgene, often integrated, into some or all of the cells of the mammal's tissues (Federoff Declaration ¶ 11). The present invention does not depend on whether all or some of the mammal's cells are transgenic given that any cells which contain a recombinatorial transgene are suitable for the introduction of cre recombinase (Id.). Application of the present invention to humans holds the potential to treat and perhaps cure individuals with various recessive disorders (Id.).

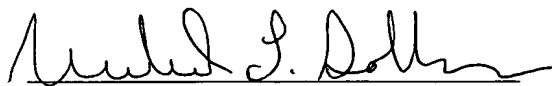


Based on the Federoff Declaration, it is apparent that the present application fully satisfies the requirements of 35 U.S.C. § 112 (1<sup>st</sup> para.). Therefore, the lack of enablement rejection should be withdrawn.

In view of all the foregoing, it is submitted that this case is in condition for allowance and such allowance is earnestly solicited.

Respectfully submitted,

Date: July 2, 2004



Michael L. Goldman  
Registration No. 30,727

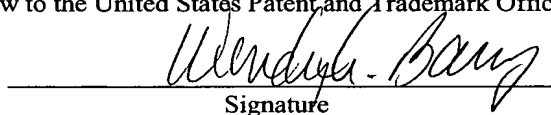
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CERTIFICATE OF MAILING OR TRANSMISSION [37 CFR 1.8(a)]

I hereby certify that this correspondence is being:

- ☒ deposited with the United States Postal Service on the date shown below with sufficient postage as first class mail in an envelope addressed to: Mail Stop RCE, Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450
- ☐ transmitted by facsimile on the date shown below to the United States Patent and Trademark Office at (703) \_\_\_\_\_.

July 2, 2004  
Date

  
Signature

Wendy L. Barry  
Type or Print Name



PATENT

Docket No.: 176/60088 (6-11406-600)

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Howard Federoff	)	Examiner:
			)	D. Crouch
Serial No.	:	09/854,869	)	
Cnfrm. No.	:	9948	)	Art Unit:
			)	1632
Filed	:	May 14, 2001	)	
For	:	PRODUCTION OF SOMATIC MOSAICISM	)	
		IN MAMMALS USING A	)	
		RECOMBINATORIAL SUBSTRATE	)	

## DECLARATION OF HOWARD FEDEROFF UNDER 37 C.F.R. § 1.132

## Mail Stop RCE

Commissioner for Patents

P.O. Box 1450

Alexandria, VA 22313-1450

Dear Sir:

I, Howard Federoff, pursuant to 37 C.F.R. § 1.132, declare:

1. I received a B.A. degree in 1974 from Earlham College, Richmond, Indiana, an M.S. degree in 1977, a Ph.D. degree in 1979, and M.D. degree, all from Albert Einstein College of Medicine, Bronx.
2. I am the Senior Associate Dean for Basic Research, a Professor of Neurology, Medicine, Microbiology, and Immunology, and a Professor of Oncology and Genetics, all at University of Rochester School of Medicine, Rochester, N.Y. and the Director for the Center for Aging and Developmental Biology, Aab Institute of Sciences, University of Rochester, Rochester, N.Y. A copy of my Curriculum Vitae is attached as Exhibit 1.
3. I am the sole inventor of the above patent application.
4. I am presenting this declaration to demonstrate that skilled scientists working in the field would recognize that my present invention could be used in conjunction with transgenic mammals and chimeric mammals for therapeutic benefit.
5. The term "transgenic" refers to the presence a foreign gene in a cellular context where it can be carried either as an integrated DNA segment or as an episome. Cells

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that harbor an integrated foreign piece of DNA, therefore, can be considered to be transgenic. (Gordon et al., "Integration and Stable Germ Line Transmission of Genes Injected into Mouse Pronuclei" *Science* 214:1244-1246 (1981) (attached as Exhibit 2); Pinkert, In *Transgenic Animal Technology: A Laboratory Handbook* pps 3-12 (2002) (attached as Exhibit 3)).

6. In the context of mammals, the most common usage of the term "transgenic" pertains to mammals that carry stably integrated copies of a gene of interest and which are conveyed to their progeny through the germ line. During the construction of conventional transgenic animals, the male pronucleus of a fertilized egg is injected with DNA (Palmiter et al., "Differential Regulation of Metallothionein-thymidine Kinase Fusion Genes in Transgenic Mice and Their Offspring," *Cell* 29(2):701-10 (1992) (attached as Exhibit 4). That DNA has the opportunity during the next several cell divisions to undergo integration into one or several chromosomal locations. After transfer of that injected egg into a pseudo pregnant mother, offspring are born that carry the integrated transgene. The transgene is often found in most, if not all, of the cells including the germ line of the founder animal. Founders need not carry transgenes in every cell and, therefore, would be considered on that basis to be chimeric (Wagner et. al., "Genetic Engineering of Laboratory and Livestock Mammals," *J Anim Sci.* 61 Suppl 3:25-37 (1985) (attached as Exhibit 5); Lim et. al., "High Level, Regulated Expression of the Chimeric P-enolpyruvate Carboxykinase (GTP)-bacterial O6-alkylguanine-DNA Alkyltransferase (ada) Gene in Transgenic Mice," *Cancer Res.* 50(6):1701-8(1990) (attached as Exhibit 6)).

7. Chimeric mammals, carrying a recombinatorial substrate in a subset of cells, would for the purposes of my present invention be suitable substrates for the delivery of cre recombinase. If the transgene in the chimeric founder animal is a recombinatorial substrate, it could support cre recombinase-mediated recombination and, following successful recombination, would express only in cells and tissues capable of recognizing the promoter driving its expression. The more frequent usage of chimeras refer to those derived from the construction of mammals where an embryonic stem (ES) cell is typically engineered by homologous recombination to harbor a loss or conditional loss of function mutation. These cells are then used to produce a transgenic mammal by injection of genetically altered ES cells into blastocysts. When these embryos are transferred to a pseudo pregnant mother, they give rise to chimeras. As in the pronuclear injection example, not every cell will harbor the transgene. Typically if those cells that harbor the transgene include the germline, there is an

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opportunity for it to be conveyed to the progeny of the mammal (Thomas et. al., "Site-directed Mutagenesis by Gene Targeting in Mouse Embryo-derived Stem Cells." *Cell* 51(3):503-12 (1987) (attached as Exhibit 7)).

8. With regard to my present invention, no distinction need be made between a transgenic mammal in which the transgene is found in all cells versus those mammals where the transgene might be found in a more circumscribed set of cells. To practice my present invention, cre recombinase can be delivered to either a founder mammal derived from pronuclear injection or a mammal derived from the introduction of genetically manipulated ES cells into the blastocyst. Thus, my present invention can be practiced independently of the source or nature of the transgene in the mammal in which the transgene is carried.

9. My present invention has potential human therapeutic importance and was indeed a driving force for its creation. Unfortunate individuals with inborn errors of metabolism (e.g., Mucopolysaccharidoses, Canavan's Disease, Hurler's Disease, Tay-Sachs, among many others) and those with later-onset recessive mutations producing devastating clinical complications (e.g., Juvenile Batten's Disease, etc.) would be suitable recipients for therapy based on my present invention. This group of disorders share the characteristic that the replacement of the missing and mutated enzyme can be accomplished in a subset of cells and that these gene-modified cells provide sufficient enzymatic function through 'cross-correction' to ameliorate the clinical phenotype. Such cross-correction can be achieved by administration of gene-modified bone marrow-derived cells (Gasper et. al., "Correction of Feline Arylsulphatase B Deficiency (Mucopolysaccharidosis VI) by Bone Marrow Transplantation," *Nature* 312(5993):467-9 (1984) (attached as Exhibit 8); Wolfe et. al., "Reversal of Pathology in Murine Mucopolysaccharidosis Type VII by Somatic Cell Gene Transfer," *Nature* 360(6406):749-53 (1992) (attached as Exhibit 9); Hoogerbrugge et. al., "Correction of Lysosomal Enzyme Deficiency in Various Organs of Beta-glucuronidase-deficient Mice by Allogeneic Bone Marrow Transplantation," *Transplantation* 43(5):609-14 (1987) (attached as Exhibit 10)).

10. However, this same enzymatic correction could be accomplished by other somatic cells genetically engineered to provide the missing enzyme. This principle underlies the therapeutic practice of my present invention. For example, an individual diagnosed with one of these disorders would have a somatic cell population engineered to carry a recombinatorial substrate that would include an inactive form of the missing enzyme. The cells to be modified with the recombinatorial substrate could be bone marrow-derived,

- 4 -

precursors or skeletal muscle cells, as non-limiting examples for discussion purposes. Depending on the particular disease, it will be necessary to titrate the amount of replacement enzyme made available to effectively reverse the clinical phenotype. This would be achieved by the introduction of cre recombinase to a fraction of the cell type(s) harboring the recombinatorial substrate and should result in the expression of the missing enzyme. The ability to titrate recombination will allow for precise control of the amount of enzyme that is to be replaced. Given that in some cases the gene-corrected cells, produced by cre-mediated recombination, can migrate and marginate (e.g., bone marrow derived macrophages can enter the brain and other organs), it is likely that wide-spread correction of the clinical response would be observed.

11. In summary, transgenic mammals are those that have a transgene, often integrated, into some or all of the cells of the mammal's tissues. My present invention does not depend on whether all or some of the mammal's cells are transgenic given that any cells which contain a recombinatorial transgene are suitable for the introduction of cre recombinase. Application of my present invention to humans holds the potential to treat and perhaps cure individuals with various recessive disorders.

12. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date:

6/30/2004

Howard Federoff  
Howard Federoff

June 2004

## **CURRICULUM VITAE**

**NAME:** Howard Joshua Federoff

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**EMAIL:** Howard\_Federoff@urmc.rochester.edu

**DATE OF BIRTH:** March 24, 1953

**SOCIAL SECURITY NUMBER:** 052-40-5768

**CITIZENSHIP:** U.S.A.

### **EDUCATION**

1974 B.A. Earlham College, Richmond, IN  
1977 M.S. Albert Einstein College of Medicine, Bronx, NY  
1979 Ph.D. Albert Einstein College of Medicine, Bronx, NY  
1983 M.D. Albert Einstein College of Medicine, Bronx, NY

### **POSTGRADUATE TRAINING**

1983-1984 Intern in Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA  
1984-1985 Resident in Medicine, Massachusetts General Hospital, Harvard Medical School Boston, MA  
1985-1986 Clinical Fellow in Endocrinology, Massachusetts General Hospital, Harvard Medical School, Boston, MA  
1986-1988 Clinical and Research Fellow in Endocrinology, Massachusetts General Hospital, Harvard Medical School Boston, MA

### **BOARD CERTIFICATION**

1987 Internal Medicine  
1989 Endocrinology & Metabolism

### **LICENSURE**

1983-1988 Licensed in Commonwealth of Massachusetts

June 2004

1988-Present Licensed in New York State

#### **ACADEMIC APPOINTMENTS**

1988 Attending Physician, Baker Medical Service, Massachusetts General Hospital, Harvard Med. School, Boston, MA  
1988-1995 Attending Physician, Jacobi Hospital and Weiler Hospital, Bronx, NY  
1988-1995 Consulting Endocrinologist, Jacobi Hospital and Weiler Hospital, Bronx, NY  
7/88-6/1993 Assistant Professor of Medicine and Neuroscience, Albert Einstein College of Medicine, Bronx, NY  
7/93-4/1995 Associate Professor of Medicine and Neuroscience, Albert Einstein College of Medicine, Bronx, NY  
5/95-Present Professor of Neurology, Medicine, Microbiology and Immunology, University of Rochester School of Medicine, Rochester, NY  
5/95-8/2003 Chief, Division of Molecular Medicine and Gene Therapy, University of Rochester School of Medicine, Rochester, NY  
6/96-Present Professor of Oncology and Genetics, University of Rochester School of Medicine, Rochester, NY  
2/97-9/2001 Director, University of Rochester Interdepartmental Neuroscience Program, University of Rochester, Rochester, NY  
5/98-Present Director, Center for Aging and Developmental Biology, Aab Institute of Biomedical Sciences, University of Rochester, Rochester, NY  
10/02-present Senior Associate Dean for Basic Research, University of Rochester School of Medicine, Rochester, NY

#### **AWARDS AND HONORS**

1974-1978 USPHS T32  
1979 – 1983 American Cancer Society Special Postdoctoral Award  
1982 Alpha Omega Alpha Election  
1997 Arthur Kornberg Research Award, University of Rochester  
1997 Inaugural Annual Brain Marrow Project Lecture, Memphis TN, 11/97  
1998 Who's Who in America  
1999 Who's Who in the World  
2003 Grass Lecturer, Kansas City Chapter of the Society for Neuroscience

#### **PROFESSIONAL MEMBERSHIP**

American Association for the Advancement of Science  
New York Academy of Science  
Society for Neuroscience  
American Society of Gene Therapy

#### **EDITORIAL BOARDS**

1999- Present *Brain and Mind*  
1999- Present *Experimental Neurology*  
2000- Present *Gene Therapy Reviews*  
2000- Present *Gene Therapy*  
2002- Present *NeuroRx*

June 2004

## **CORPORATE INTERACTIONS**

Consultant, Promega Corporation 1996-2000

Founding Scientist, Socratech, LLC, 2000

Consultant, Integrated Nano-Technologies, LLC, 2000 - present

Consultant, Abbott Pharmaceuticals, 2001

Consultant, Avigen, Inc., 2001-2003

Founding Scientist, AmpliVex, LLC, 2002-present

## **SPECIAL COURSES**

1977 - Cold Spring Harbor Laboratory, Advanced Bacterial Genetics

1986 - Marine Biological Laboratory, Woods Hole, Neurobiology

1992 - Cold Spring Harbor Laboratory, Mouse Embryology

## **TEACHING EXPERIENCE**

1988-1989 Attending Physician, Baker Medical Service, Massachusetts General Hospital

1989-1995 Attending Physician, Medical Service BMHC Hospital

1989-1995 Attending Physician, Endocrinology, BMHC Hospital

1992-1995 Director and Lecturer, Section on Neural Development, Graduate Neuroscience course, Albert Einstein College of Medicine

1995 Lecturer, Cell Signaling, Department of Pharmacology, University of Rochester School of Medicine and Dentistry

1996 Lecturer, Neurobiology of Disease, Department of Neurobiology and Anatomy, University of Rochester School of Medicine and Dentistry

1996-present Lecturer, Medical Genetics

1997-present Lecturer, Endocrine Physiology, University of Rochester School of Medicine and Dentistry

1997-present Lecturer, Cellular Neuroscience, University of Rochester School of Medicine and Dentistry

1998-1999 Lecturer, Principles of Behavior Analysis, University of Rochester School of Medicine and Dentistry

1998-present Lecturer, Toxicology Core Course, University of Rochester School of Medicine and Dentistry

2001- Instructor, Ph.D. Readings in Neuroscience, University of Rochester School of Medicine and Dentistry

2001- Lecturer, Neuroscience Investigative Seminars, Department of Neurosurgery

2001- Lecturer, MBB II: Advanced Basic Sciences, Development & Degeneration: A Life-

Long Balance Influencing Brain Function

## **COMMITTEE SERVICE**

### **Local**

1989 - 1995 Steering Committee, Medical Scientist Training Program, Albert Einstein College of Medicine

1995-1997 University of Rochester Medical Center Strategic Planning Committee

1995-1997 University of Rochester Medical Center Strategic Implementation Committee, Aging and Development

1995-Present Neurology Executive Committee



June 2004

1995 Search Committee, Chief, Pediatric Hematology-Oncology, Department of Pediatrics  
1995-1996 Graduate Student Admissions Committee, Department Microbiology and Immunology  
1996 Chair, Thesis Defense, D. Luan, Department of Biology  
1996 Chair, Qualifying Committee for Jeffrey Rumbaugh, Department of Biochemistry  
1996-1998 Thesis Committee for Jeffrey Rumbaugh, Department of Biochemistry  
1996-Present Users Committee, URM C New Building Design  
1997 Thesis Committee for Derek Choi-Lundberg, Dept. of Neurobiology & Anatomy  
2000 Thesis Committee for Jing Niu, Department of Biology  
1997-1999 Thesis Committee for Ganesan Satya, Department of Biochemistry and  
Biophysics  
1996-1998 Search Committee, Chair, Department of Biochemistry and Biophysics  
1997-1998 Search Committee, Chair, Department of Environmental Medicine  
1997-Present Committee for MD Degree with Research Distinction  
1997-1998 Search Committee, Directors for Vaccine Biology and Cancer Centers  
1999-2000 Search Committee, Department of Ophthalmology  
1999-present Executive Committee of the Schmitt Program on Integrative Brain Research  
1999-present Graduate Education in the Basic Sciences/Basic Science Depts./Research Committee  
1999-present Chair, Users' Committee of the Transgenic Mouse Core Facility  
2000-2001 Search Committee, Vivarium Director, Department of Laboratory and Animal Medicine  
2000-2003 Search Committee, Chief Endocrinology, Department of Medicine  
2000 Rand Corporation  
2000 Search Committee, Chair, Department of Neurosurgery  
2001 Thesis Committee for Liz Lipscomb, Department of Toxicology  
2002 Thesis Committee for Chiayu Chiu, Neuroscience Graduate Program  
2002- Search Committee, Chair, Department of Biomedical Genetics  
2002 Chair, Qualifying Committee for Michael Froehler, Neuroscience Graduate Program  
2002 Thesis Committee for Chiawen (Kitty)Wu Neuroscience Graduate Program  
2003 Search Committee, Translational Physician Scientists, Cancer Center  
2003 Board Member, Institutional Biosafety Committee (IBC), University of Rochester  
2003-present Search Committee, Department of Medicine, Human Genetics Program  
2004-present University Advisory Committee for the Presidential Search  
**National**  
1996-1999 Member, NLS3 Study Section  
1999-2003 Chair, BDCN3 Study Section  
2000-2001 NIH MDCN IRG Working Group  
2000-present Board of Scientific Counselors, NIDCR  
2001-present Member Neurologic disease committee, ASGT  
2000-2003 American Academy of Neurology: Ethics, Law and Humanities Committee  
2001, 2002 *ad hoc* reviewer, Recombinant DNA Advisory Committee  
2001- Board Member, American Federation of Aging Research  
2003-2003 Chair, CDIN Study Section (*formerly BDCN-3*)  
2003- present Member, NINDS Spinal Muscular Atrophy Steering Committee  
2004 Member, Search Committee, NIDCR Scientific Director  
2004- present Member, vector committee, ASGT

## **PEER REVIEWER**

Journals Brain Research

June 2004

Experimental Neurology  
Gene Therapy  
Human Gene Therapy  
J. Neuroscience  
Lancet  
Lung  
Nature Biotechnology  
Nature Genetics  
Nature Medicine  
Neurobiology of Disease  
Neurobiology of Aging  
Neuroscience  
Neuroscience Letters

Grants

Proceedings National Academy of Science  
NSF, Neuronal and Glial Mechanisms, 1994 - 1997  
Spinal Cord Research Foundation  
NIH, NSRA, MHAI-2, Special Reviewer, 1995  
Cystic Fibrosis Foundation- Gene Therapy  
NIH, Neurology C Study Section, *Ad hoc* 2/95  
Fighting Blindness Foundation, 6/96  
Alzheimer's Association, 1997, 1998, 1999  
NIMH, Special Emphasis Panel, 1997  
Canadian MRC Centers of Excellence Program in Neuroscience, 1998  
NIH, Neurological Sciences III, Standing member 6/95 - 2/98  
NIH BDCN4, Adhoc 6/98- 2/99  
Department of Defense, NETRP, 1997- 1998  
ALS Society, 1999  
NIH BDCN3, Chair 6/99-7/03  
Danish National Research Foundation, 2000-present  
The Jacob and Valeria Langeloth Foundation  
Institute Brain Research and Dementia, University of California at Irvine  
NIH CDIN, Chair 7/03-present (*formerly BDCN-3*)

**TRAINEES**

Predoctoral: Bing Lu, M.D., Ph.D. candidate, 1992 - 1994  
Alborz Hassankhani, MSTP, 1991- 1995  
Michael Geschwind, MSTP, 1991- 1995  
Adriana Rozental, Ph.D. candidate, 1992- 1995  
Robert Starr, MSTP, 1992 - 1995  
Andrew Brooks, Ph.D. candidate, 1994 – 2000  
Marc Halterman, MSTP, 1995- 2002  
Michael Derby, Ph.D. candidate, 1996 - 1997  
Keith Barlow, Ph.D. candidate, 1996 - 1997  
Joe Sanchez, Ph.D. candidate, 1996 - 1999  
Brandon Harvey, Ph.D. candidate, 1996 – 2003  
Renee Miller, Ph.D. candidate, 1999 – present  
Yu (Agnes) Luo, Ph.D. candidate, 1999-present

June 2004

Jason Hamilton, Ph.D. candidate, 2000-present  
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Charles Wuertzer, Ph.D. candidate, 2000-present  
Kuei-Cheng Lim, MSTP candidate, 2000-present  
Michelle Janelins, Ph.D. candidate, 2003-present

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Peter Zahos, M.D., 1993 – 1994  
Nariman Panahian, M.D., Ph.D., 1995 - 1997  
William Bowers, Ph.D., 1995 – 1998  
Timothy Corden, M.D., 1995 – 1996  
Kathleen Maguire-Zeiss, Ph.D., 1996 - 2002  
Hui Huang, Ph.D., 1997 – 2000  
Craig Miller, M.D., Ph.D., 1998 – 1999  
Eric Detrait, Ph.D., 1999-2001  
Stephanos Kyrkanides, D.D.S., Ph.D., 1999 – 2000  
Xiaowei Chen, M.D., Ph.D., 1999-2001  
Seung Lim, Ph.D., 2001-2003  
David Rempe, M.D., Ph.D., 2001-2003  
Feng Xing, Ph.D., 2003-

#### **PATENTS**

US 6,051,428: Rapid production of autologous tumor vaccines  
Inventors: Y. Fong, H. J. Federoff, and J. D. Rosenblatt  
Issued: April 18, 2000

US 5,156,306: Pancreatic beta-cells for allogeneic transplantation without immunosuppression  
Inventors: M. Brownlee, M. Horowitz, H.J. Federoff and S. Efrat  
Issued: December 5, 2000

US 6,252,130: Production of Somatic Mosaicism in Mammals Using a Recombinatorial Substrate  
Inventor: H.J. Federoff  
Issued: June 26, 2001

PCT/US01/16682  
Filing date: 5/23/00  
Title: "Method for Producing HSV Amplicons and Uses Thereof"  
Inventors: H.J. Federoff and W.J. Bowers

US Utility 09/997,848  
PCT/US01/47808  
Filing date: 11/29/01  
Title: "Helper Virus-Free Herpes Virus Amplicon Particles and Uses Thereof"  
Inventors: H.J. Federoff, W.J. Bowers, S. Dewhurst, T. Evans, J. Frelinger, R. Willis, K. Tolba, and J. Rosenblatt

June 2004

PCT/US02/01069

Filing date: 1/18/02

Title: "Central Role of Brain Endothelium in Pathogenesis of Alzheimer's Disease"

Inventors: B. Zlokovic, H.J. Federoff

PCT/US03/05712

Filing Date: 2/25/03

Title: "DNA Construct for Inducible Expression of Angiogenic Protein"

Inventors: H.J. Federoff and W. J. Bowers

PCT/US03/17318

Filing Date: 5/31/03

Title: "Integrated HSV Amplicon Vector"

Inventors: H. J. Federoff and W. J. Bowers

Provisional 60/442,303

Filing Date: 1/23/03

Title: "Herpes Amplicon Particles"

Inventors: H.J. Federoff, W.J. Bowers, and M.W. Halterman

Provisional 60/480,112

Filing Date: 6/20/03

Title: "Prevention of Treatment of Deficits that Arise in Connection of or Injuries to the Nervous System"

Inventors: H.J. Federoff, W.J. Bowers, V. Arvanion, and L. Mendell

Provisional 60/518,474

Filing Date: 11/07/03

Title: Compositions and Methods of Treating Neurological Disorders

Inventors: H. J. Federoff and W. J. Bowers

#### **INVITED PRESENTATIONS 1995 – Present**

"Gene Therapeutic Approaches for Neuronal Salvage", Gene Therapy of Central Nervous Disorders University of Pennsylvania, Philadelphia, PA 6/95

"Genetic Therapy", NIH Parkinson's Disease Research Planning Workshop, Washington, DC 8/95

"Gene Transfer in Neurobiology", European Neuroscience Meeting, Amsterdam, NL 9/95

"Selective Cardiac Overexpression of NGF in Transgenic Mice", University of Leiden, Leiden, NL 9/95

June 2004

“Cellular and Molecular Treatments of Neurologic Diseases”, Harvard University, Cambridge, MA 10/95

“Network Modification”, Neurotrophic Factors in Development, Plasticity and Survival, Madison, WI 10/95

“Gene Transfer into the nervous system: implications for disease pathogenesis”, Annual Meeting of the American Neurological Association, Washington, D.C. 10/95

“Somatic and Germline Approaches for Neurotrophin Manipulation”, University of Pittsburgh, Department of Molecular Biology, Pittsburgh, PA 10/95

“Manipulation of a Network: Gene Targeting in Development “, Genova, Italy 3/96

“Neuronal Salvage”, The First Meeting of the Parkinson’s Disease Gene Therapy Consortium, Washington, DC, 4/96

“Genetics and the use of biomaterials” Moving into the 21st century: Frontiers in human tissue research, Philadelphia, PA, 4/96

“Gene Transfer: Applications of Viral Vectors for the Study and Treatment of CNS Disorders”, Co-Chair, Symposium , Society for Neuroscience Meeting, Washington, DC, 11/96.

“Somatic Mosaic Analysis of NGF Function” Winter Conference on Brain Research, 1/97

“Somatic and Germline Manipulation of Neurotrophin Function” University of Iowa, 4/97

“Gene Therapy for Parkinson’s disease” United States House of Representatives Subcommittee on Appropriations” Washington, DC, 6/97

“Manipulation of NGF Function *in vivo*” Tufts University School of Medicine, 6/97

“Strategies to Ameliorate Neuron Death” 8th International Symposium on Stroke, Neurotrauma and Other Neurological Diseases” New Orleans, LA 7/97

“Gene Therapy for Neurologic Diseases” Promega Consultants Symposium, Madison, WI, 7/97

“Direct CNS Gene Transfer for Reduce Neuron Death” European Neuroscience Summer School, Amsterdam, NL, 8/97

“Gene Transfer into the Central Nervous System: An Experimental Tool and a Potential Therapy”, First Annual Brain Marrow Project Lecture, Memphis TN, 11/97

“Gene Transfer into the CNS” WCBR, UT, 1/98

June 2004

“Perspectives in Neuroscience - Manipulation of NGF Expression within the Murine CNS”,  
“Developing Gene Therapy for Neurological Diseases”, 2 Lecture Series, Clinical Neurological  
Science Rounds, London, Ontario, 4/98

“HSV Gene Transfer: An Experimental Tool and Potential for Therapy”, University of Toronto -  
Mount Sinai Hospital, Toronto Ontario, 4/98

“Viral Vectors”, Fifth Annual Conference of the American Society for Neural Transplantation,  
Clearwater, FL, 4/98

“Introduction of Concept Clearance in Developmental Neurotoxicology and Neurodegenerative  
Diseases”, Ninety-Fourth Regular Meeting of the National Advisory Environmental Health  
Sciences Council, NIH/NIEHS, Bethesda, MD, 5/98

“Gene Transfer to the Nervous System: Experimental Tool and Potential for Therapy”,  
Symposium for Gene Expression in the Nervous System, Harvard Medical School, Boston, MA,  
5/98

“Somatic mosaic analysis of NGF function in the CNS”, Molecular Biology Seminar Series,  
University of Kansas, Lawrence, KS, 5/98

“Gene delivery and gene therapy methodologies for CNS applications”, 2nd Cellular and  
Molecular Treatments of Neurological Diseases Conference, American Academy of Arts and  
Sciences, Cambridge, MA, 10/98

“Somatic Mosaic Analysis in Mice: An Approach to Study Gene Product Function in the CNS”,  
A Satellite Symposium to the 1998 Society for Neuroscience Annual Meeting, Sponsored by  
NIAAA, NIH, Los Angeles, CA 11/98

“Genetic Modifications of the Brain: Strategies to Elucidate Function”, Neuroscience  
Colloquium, University of Rochester, Rochester, NY, 2/99

“CNS Gene Transfer with HSV Vectors”, Promega Corporation, Madison, WI, 4/99

“Probing the Function of NGF in the Adult CNS by Somatic Mosaic Analysis”, Neuroscience  
Lecture, University of Wisconsin-Madison, Madison, WI, 4/99

“Hypoxic Signaling in Neurons”, Chair, “Signal Transduction Session”, First Gordon Conference  
on NeuroVirology, Seminar: “Adaptive and Pathophysiologic Signaling in Hypoxia”, Colby  
Sawyer College, New London, NH, 6/99

“CNS Gene Transfer to Modify Learning”, The American Society of Gene Therapy, Second  
Annual Meeting, Washington, DC, 6/99

“Approaching Gene Transfer to the Nervous System from the Inside and Out”, Winter  
Conference on Brain Research, Breckenridge, CO, 1/00

June 2004

“CNS Gene Transfer to Modify Learning”, University of Connecticut Health Center, Farmington, CT, 2/00

“Gene Transfer to Modify Learning”, University of Pennsylvania Health System, Philadelphia, PA, 4/00

“CNS Gene Transfer: Experimental Tool and Potential for Therapy”, Neurology Grand Rounds, Johns Hopkins University, Baltimore, MD, 4/00

“Gene transfer to modify the neural substrate underlying learning and memory”, Cells and Genes and Their Applications for Therapies for the Brain, FASEB, San Diego, CA 4/00

“Applications of HSV Vectors for Experimental Neurobiology”, NIH, NIDA, 5/00

“Methodologies for manipulating single genes in the adult”, American Society of Gene Therapy 3<sup>rd</sup> Annual Meeting, Denver, CO 6/00

“Gene-experience interaction alters the cholinergic septohippocampal pathway of mice”, The Year 2000 Schmitt Symposium, University of Rochester, Rochester, NY, 8/00

“Genes, Environment, and Aging: Interaction and Involvement in Disease”, Symposium on Aging, 75<sup>th</sup> Anniversary of the University of Rochester Medical Center, Rochester, NY, 10/00

“Gene Transfer to the Nervous System: Status and Promise for Therapy”, Third Annual Meeting of the American Society for Experimental Neurotherapeutics, Washington, DC, 3/01

“Summary Report”, Workshop on DoD Sponsored Parkinson’s Related Research, Bolger Center, Potomac, MD, 3/01

“Gene Therapy: Scientific, Ethical and Regulatory Issues”, 53<sup>rd</sup> Annual Meeting of the American Academy of Neurology, Philadelphia, PA 5/01

“Manipulation of genes to further our understanding and treatment of disease”, Washington Science Writers Seminar: Emerging Technologies & Interventions for Brain Repair, Washington, DC 5/01

“Modes of Gene Delivery and Expression in the CNS”, 4<sup>th</sup> Annual American Society of Gene Therapy, Seattle, WA 5/01

“Amplicon vector gene transfer to evaluate nervous system function”, 4<sup>th</sup> Annual American Society of Gene Therapy, Seattle, WA 5/01

“Principles of CNS gene therapy”, Bench science research: implications for treatment of TBI, National Brain Injury Association 20<sup>th</sup> Annual Symposium, Atlanta, GA 7/01

“Bionomics analysis of hypoxic injury”, Pediatrics Travel Club, Rochester Academy of Medicine, Rochester, NY 9/01

June 2004

“Probing the aging CNS functions by gene transfer”, Brain Aging-Identifying Accelerators and Brakes, San Diego, CA 11/01

“Evolving Perspectives on CNS Gene Therapy”, Winter Conference on Brain Research, Aspen, CO, 1/02

“Experience, Plasticity and the Aging Brain”, Adler Foundation Symposium, Salk Institute, Torrey Pines, CA, 1/02

“Molecular dissection of pathologic mechanisms: Integrative Bionomics”, Cellular and Molecular Treatments of Neurological Diseases Conference, American Academy of Arts and Sciences, Cambridge, MA, 3/02

“Molecular Mechanisms of Alzheimer’s Disease”, Case Seminar in Aging, Monroe Community Hospital, Rochester, NY, 4/02

“Stem Cells: Biologic and Ethical Issues”, University of Rochester, Rochester, NY 4/02

“Plasticity and the Aging Brain”, William Hall Symposium, University of Rochester, Rochester, NY, 5/02

“A Celebration of Science: The Healing Power of Knowledge”, Harvard Club of New York City, NY 5/02

“Molecular Genetic Manipulation of the Adult Central Nervous System”, Workshop on “Aging in the Nervous System”, University of Michigan, Ann Arbor, MI 5/02

“Dissection of Neurologic Disease Mechanisms by Gene Transfer”, Scientific Symposium, 5<sup>th</sup> Annual American Society of Gene Therapy, Boston, MA 6/02

“Gene Transfer Strategies for Treatment of Neuromuscular Disorders”, Corporate Symposia, 5<sup>th</sup> Annual American Society of Gene Therapy, Boston, MA 6/02

“Evolution of Gene Therapy for Parkinson's Disease”, Annual Parkinson’s Disease Symposium, Radisson Inn, Rochester, NY 9/02

“Response to injury at the Cellular Level: Defense and Compensation”, Parkinson’s Disease: The Life Cycle of the Dopamine Neuron, A New York Academy of Sciences Conference, Princeton, NJ 9/02

“CNS Diseases Amenable for Gene Therapy”, Ernst Schering Research Foundation Workshop 43 - Human Gene Therapy: Current Opportunities & Future Trends, Berkeley, CA 10/02

“Genetic Approaches to Study CNS Function and Repair”, Schmitt Program on Integrative Brain Research Symposium - Cellular Approaches to the Understanding of CNS Development, Damage and Repair, University of Rochester, 10/02



June 2004

“NGF: Constitutive and Activity Dependent Modulator of Synaptic Function”, 3<sup>rd</sup> Neurobiology of Aging Conference, Orlando, FL 10/02

“A Proteomic Approach for Potential Biomarker Identification”, Proteomics and Aging Workshop, National Institute of Aging, Bethesda, MD 12/02

“Molecular Mechanisms of Alzheimer’s Disease”, Case Seminar in Aging, Monroe Community Hospital, Rochester, NY, 4/03

"Gene Therapy: Current Reality and Future Prospect for Parkinson's Disease", Mercer University of Medicine, Macon, GA 4/03

"Molecular Medicine: Its General Principles and Applications", Core Curriculum Seminar in Internal Medicine, Mercer University of Medicine, Macon, GA 4/03

“Gene Therapy – GDNF”, Scientific Overview Panel, 9<sup>th</sup> Annual Pan Forum Research and Education Forum and Public Policy Forum, Parkinson’s Action Network, Washington, DC 5/03

“Molecular Genetic Manipulation of the CNS Elucidating Function an Approaching Therapies”, Kansas City Chapter of the Society for Neuroscience as a Grass Traveling Scientist, University of Kansas Medical School, Kansas City, KS 5/03

“Central Nervous System Gene Transfer”, Education Program of the American Society of Gene Therapy 6<sup>th</sup> Annual Meeting, Washington, DC 6/03

“Neurodegenerative Disease and Proteomics: Strategies for Therapeutic Discovery”, The Biotechnology Industry Organization (BIO), 2003 Annual Convention, Washington, DC 6/03

“Loosening the Grip of Parkinson’s Disease”, Medical School for an Evening: ‘Progress: From the Bench to the Bedside’, University of Rochester, Rochester, NY 6/03

“The Aging Brain and its Diseases”, Project Medical Education, “Research Rotations”, University of Rochester, Rochester, NY 8/03

“HSV vector –mediated gene delivery”, Plenary Speaker for the 5th International Symposium on NeuroVirology, Renaissance Harborplace Hotel, Baltimore, MD 9/03

“Molecular approaches to unraveling neurodegenerative diseases: implications for early diagnosis and novel therapy development”, Amersham Biosciences, Piscataway, NJ 10/03

“Gene Transfer for the CNS: An Experimental Tool and Potential for Therapy”, Amgen, Inc., Thousand Oaks, CA 11/03

“NAD as an Integrative Sensor: Linking Cellular Energy Metabolism and Cell Death”, Nathan Shock Center Symposium, San Diego, CA 11/03

“Virus Vectors to Dissect CNS Function and Develop New Therapy”, University of California, Irvine, Irvine, CA 11/03

June 2004

"Molecular Mechanisms of Alzheimer's Disease", Case Seminar in Aging, Monroe Community Hospital, Rochester, NY 3/04

"The Future of CNS Gene Therapy", Neurology Grand Rounds, Johns Hopkins University, Baltimore, MD, 4/04

"Mechanisms underlying MPTP injury to the mouse substantia nigra as revealed by microarray analysis", CodeLink North American VIP Event, Chandler AZ. Sponsored by Amersham Biosciences 5/04

"Nurr1: Downstream targets and implications for Parkinson's Disease", Neuronal Cell Differentiation and Development at Normal and Disease Stages Workshop, Temple University, Philadelphia, PA 5/04

"HSV Amplicons for Vaccination", Neural Disorders: The Neuroimmunology of Gene Therapy Session, American Society for Gene Therapy 7th Annual Meeting, Minneapolis, MN 6/04

"Redefining gene-based neuroprotective strategies", New Directions in Neuroprotection: Basic mechanisms, Molecular Targets and Treatment Strategies Workshop, New York Academy of Sciences, New York, NY 6/04

"Novel Gene Therapeutic Strategies for Neurodegenerative Diseases", Ernst Schering Research Foundation Symposium "Opportunities and Challenges of the Therapies Targeting CNS Regeneration, Yountville, CA 6/04

## **CURRENT RESEARCH INTERESTS:** (Keywords) *Gene Therapy, Neurodegenerative Diseases*

## **PUBLICATIONS**

Needleman, R.B., **Federoff**, H.J., Eccleshall, T.R., Buchferer, B. and Marmur, J. (1978) Purification and Characterization of an Alpha-Glucosidase from *Saccharomyces carlsbergensis*, *Biochemistry* 17(22): 4657-4661.

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**Federoff**, H.J., Cohen, J.D., Eccleshall, T.R., Needleman, R.B., Buchferer, B., Giacalone, J., and Marmur, T. (1982) The Isolation of a Maltase Structural Gene from *S. carlsbergensis*. *J. Bacteriol.* 149(3):1064-1070.

**Federoff**, H.J., Eccleshall, T.R. and Marmur, J. (1983) The Regulation of Maltase Synthesis in *S. carlsbergensis*. *J. Bacteriol.* 154(3):1301-1308.

**Federoff**, H.J., Eccleshall, T.R. and Marmur, J. (1983) Carbon Catabolite Repression of Maltase Synthesis in *S. carlsbergensis*. *J. Bacteriol.* 156(1):301-307.

**Federoff**, H.J., Grabczyk, E. and Fishman, M.C. (1988) Dual Regulation of GAP-43 Gene

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Expression by Nerve Growth Factor and Glucocorticoids. *J. Biol. Chem.* 263(36):19290-19295.

De la Monte, S.M., **Federoff**, H.J., Ng, S., Grabczyk, E. and Fishman, M.C. (1989) GAP-43 Gene Expression During Development: Persistence in a Distinctive Set of Neurons in the Mature Central Nervous System. *Dev. Brain Res.* 46(2):161-168.

Biller, B.M.K., **Federoff**, H.J., Koenig, J.L. and Klibanski, A. (1990) Abnormal Cortisol Secretion and Responses to Corticotropin Releasing Hormone in Women with Hypothalamic Amenorrhea. *J. Clin. Endocrinol. Metab.* 70(2): 311-317.

Grabczyk, E., Zuber, M.X., **Federoff**, H.J., Ng, S., Pack, A., and Fishman, M.C. (1990) Cloning and Characterization of Rat Gene Encoding GAP-43. *Eur. J. Neurosci.* 2:822-827.

Lustig, R.H., Sudol, M., Pfaff, D.W., and **Federoff**, H.J. (1991) Estrogenic Regulation and Sex Dimorphism of Growth-Associated Protein 43 kDa (GAP-43) Messenger RNA in the Rat. *Mol. Brain Res.* 11(2):125-132.

**Federoff**, H. J., Geschwind, M., Geller, A. I., and Kessler, J.A. (1992) Expression of Nerve Growth Factor *in vivo* from a Defective Herpes Simplex Virus I Vector Prevents Effects of Axotomy on Sympathetic Ganglia. *Proc. Natl. Acad. Sci. (USA)* 89(5):1636-1640.

**Federoff**, H.J., Lawrence, D. and Brownlee, M. (1993) Nonenzymatic Glycosylation of Laminin and the Laminin Peptide CIKVAVS Inhibits Neurite Outgrowth. *Diabetes* 42(4):509-513.

Lustig, R.H., Hua, P., Wilson, M.C., and **Federoff**, H.J. (1993) Ontogeny, Sex Dimorphism, and neonatal sex hormone determination of Synapse-Associated Messenger RNAs in Rat Brain. *Mol. Brain Res.* 20(1-2):101-110.

Bergold, P.J., Casaccia-Bonnet, P., Zeng, X.L., and **Federoff**, H.J. (1993) Transsynaptic Neuronal Loss Induced in Hippocampal Slice Cultures by a Herpes Simplex Virus Vector Expressing the GluR6 Subunit of the Kainate Receptor. *Proc. Natl. Acad. Sci. (USA)* 90(13):6165-6169.

Casaccia-Bonnet, P., Bendikz, E., Stelzer, A., Edelstein, D.E., Brownlee, M.D., **Federoff**, H.J., and Bergold, P.J. (1993) Localized Gene Transfer into Organotypic Hippocampal Slice Cultures and Acute Hippocampal Slices. *J. Neurosci. Methods* 50(3):341-351.

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Starr, R., Lu, B. and **Federoff**, H.J. (1994) Functional Characterization of the Rat GAP-43 Promotor. *Brain Res.* 638(1-2):211-220.

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Geschwind, M.D., Kessler, J.A., Geller, A.I., and **Federoff**, H.J. (1994) Transfer of the Nerve Growth Factor Gene Into Cell Lines and Cultured Neurons Using a Defective Herpes Simplex Vector. *Mol. Brain Res.* 24(1-4):327-335.

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Fong, Y., **Federoff**, H.J., Brownlee, M., Blumberg, D., Blumgart, L.H., and Brennan, M.F. (1995) Rapid and Efficient Gene Transfer in Human Hepatocytes by Herpes Viral Vectors. *Hepatology* 22(3):723-729.

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Geschwind, M.D., Hartnick, C.J., Liu, W., Amat, J., Van De Water, T.R., and **Federoff**, H.J. (1996) Defective HSV-1 Vector Expressing BDNF in Auditory Ganglia Elicits Neurite Outgrowth: Model for Treatment of Neuron Loss Following Cochlear Degeneration. *Hum Gene Ther* 7(2):173-182.

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Jin, B.K., Belloni, M., Conti, B., **Federoff**, H.J., Starr, R., Son, H., Baker, H., and Joh, T.H. (1996) Prolonged in vivo gene expression driven by tyrosine hydroxylase promoter in a defective herpes simplex virus amplicon vector. *Hum Gene Ther* 7(16): 2015-2024.

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Karpoff, H.M., D'Angelica, M., Blair, S.L., Brownlee, M.D., **Federoff**, H.J., and Fong, Y. (1997) Prevention of Hepatic Tumor Metastases in Rats with Herpes Viral Vaccines and  $\gamma$ -Interferon. *J. Clin. Invest.* 99:799-804.

Lu, B., **Federoff**, H.J., Wang, Y., Goldsmith, L.A., and Scott, G. (1997) Topical Application of Viral Vectors for Epidermal Gene Transfer. *J. Invest Dermatol.* 108(5):803-808.

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Brooks, A.I., Chadwick, C.A., Gelbard, H.A., Cory-Slechta, D.A. and **Federoff**, H.J. (1999) Paraquat Elicited Neurobehavioral Syndrome Caused by Dopaminergic Neuron Loss. *Brain Res* 823(1-2):1-10.

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22 June 1981

## Integration and Stable Germ Line Transmission of Genes Injected into Mouse Pronuclei

**Abstract.** Genetic material has been successfully transferred into the genomes of newborn mice by injection of that material into pronuclei of fertilized eggs. Initial results indicated two patterns of processing the injected DNA: one in which the material was not integrated into the host genome, and another in which the injected genes became associated with high molecular weight DNA. These patterns are maintained through further development to adulthood. The evidence presented indicates the covalent association of injected DNA with host sequences, and transmission of such linked sequences in a Mendelian distribution to two succeeding generations of progeny.

The successful introduction of exogenous DNA into cultured mammalian cells (1-4) has led to the development of a novel gene transfer system that has yielded new information about gene regulation in higher eukaryotes. One difficulty with this system is that cultured cells are not capable of organismal development and differentiation. DNA sequences cloned by recombinant DNA technology can be microinjected into the pronuclei of fertilized mouse oocytes and

can be subsequently located in the DNA of newborn mice (5). This system allows the study of transferred gene sequences in the context of normal embryonic development. Since development is a process that includes maturation to adulthood, reproduction, and senescence, it is important to examine the fate of transferred genes beyond the point of birth. We have now followed this injected material through further stages of mouse development.

Two recombinant plasmids were used for microinjection. The first, designated pST6 (5), was composed of the Hind III C restriction endonuclease fragment of SV40 virus and the herpes virus thymidine kinase (TK) genes cloned in plasmid pBR322; the second, pIf (6), contained human leukocyte interferon complementary DNA (cDNA) also cloned in pBR322 (7). A simplified diagram of each plasmid with its relevant restriction sites is shown in Fig. 1. Between 1000 and 35,000 copies of each plasmid were injected into each zygote. All microinjections were carried out as described (5).

The feasibility of producing such genetically transformed mice, which we call "transgenic" mice, depends upon several factors. Our experience has been that higher copy number gives a higher rate of transformation, but that the viscosity of concentrated preparations increases embryo mortality at the time of injection. Injection of 1000 copies of pST6 gave a survival rate of 50 to 70 percent with a third of the survivors eventually giving rise to live young. About 1 in 30 of such young retained transferred genes (5). When 30,000 copies of this plasmid were injected, embryo survival was reduced to 30 to 50 percent, but 1 in 15 mice retained the sequences. The pIf plasmid is smaller than pST6 and was therefore more easily injected. Survival of microinjection of 10,000 copies of this plasmid varied between 50 and 75 percent. Ten mice were born from 33 embryos thus far implanted, a rate which compares well with survival rates of embryos injected with pST6 (5). Of these ten mice, one was transgenic. This rate appears higher than that obtained from pST6 injections, but statistically significant numbers allowing a rigorous comparison of these experiments are not yet available.

Southern blot hybridization has been used to evaluate plasmid sequences in newborn and adult mice (5, 8-10). In the case of adults, DNA was extracted from spleens. Whether or not the donor material was integrated into the host genome was assessed by three criteria: (i) the acquisition of restriction sites in the host genome but not in the recombinant plasmids, (ii) the mobility of plasmid sequences in agarose gels when the DNA applied to the gels was undigested, and (iii) the ability of the plasmid sequences to be transmitted through the germ line to succeeding generations.

Two mice (73 and 9.02) injected with either pST6 or pST9 (pST9 is identical to pST6 except that the orientation of the SV40 insert is reversed) and one mouse

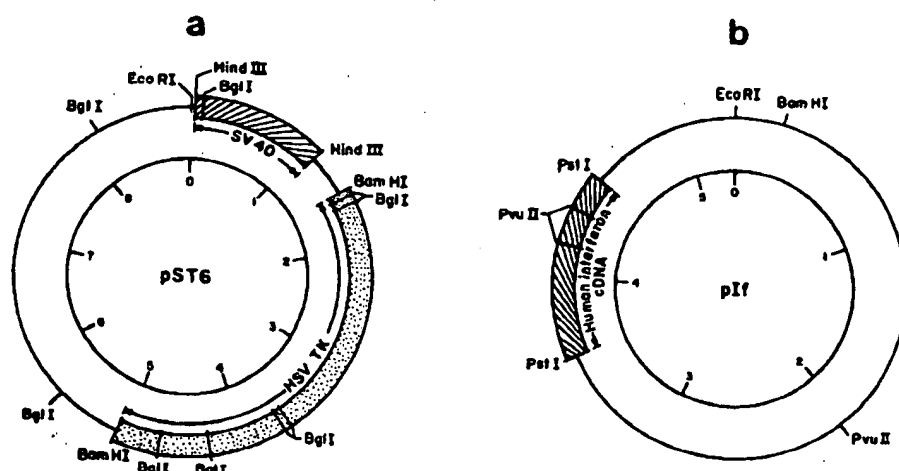


Fig. 1. Simplified diagrams of recombinant plasmids used for microinjection of mouse embryos. The Eco RI site of pBR322 is marked at 0 kb for reference.

(If-4) injected with plf retained plasmid sequences whose restriction patterns were consistent with integration. When undigested, the DNA of all three mice gave single bands of high molecular weight upon filter hybridization. This result suggests an association of the plasmid sequences with high molecular weight DNA. When cut with the restriction enzymes Xba I and Xho I, which do not recognize sites in either recombinant plasmid, DNA from mice 9.02 and 73 again gave single high molecular weight bands. The mobility of these bands could not be distinguished from each other or from that of the band produced by undigested DNA. Digests with enzymes that excise internal fragments of pST6 and pST9 showed no evidence of concatamerization of the plasmid in DNA from mice 73 and 9.02. These high molecular weight bands were thus suggestive of integration. When digested with Xba I, DNA from mouse If-4 yielded a single band of 13.5 kilobases (kb), a much larger size than the original 5.2-kb plasmid. These patterns are again consistent with integration into the host genome at a single site. However, these results do not conclusively demonstrate covalent association of the plasmid with the host DNA.

Double digests of the DNA from mice 9.02 and 73 provided additional evidence for integration. The first digest, with Bam HI, was followed by digests with Xba I or Xho I (Fig. 2). The 7.8-kb Bam HI band in mouse 73 was converted to 5.6 kb by Xba I. Similarly, the 18-kb band in mouse 9.02 was reduced to 15 kb by Xba I. These alterations in mobility indicate the acquisition of Xba I sites, a result consistent with integration.

Similar results permitted the same conclusion regarding the state of plf sequences in the spleen DNA of animal If-4. A partial digest with Xba I yielded several high molecular weight bands after hybridization with the plf probe (Fig. 3c). The smallest of these bands, 13.5 kb, was the only band produced by a complete digest with Xba I. The latter pattern obtained by partial digestion indicates linkage of the plasmid to DNA sequences containing multiple Xba I sites. The 13.5-kb band was generated by cutting at the Xba I sites closest to the point of attachment of the plasmid, whereas the larger fragments were produced when one or both of these closest sites was not digested, but more distant sites were cleaved. These results thus demonstrate for all three mice that plasmid DNA had become ligated to host genomic sequences, but they do not con-

clusively demonstrate integration into a host chromosome. A satisfactory test of this possibility is breeding of the transformed adult. Integration into a single chromosomal homolog should result in Mendelian transmission of the plasmid sequence as a heterozygous marker.

Since mice 73 and 9.02 were killed at birth, only mouse If-4 could be subjected to progeny testing.

We applied this test to the adult mouse If-4, and the results obtained were consistent with chromosomal integration of the injected plasmid. This mouse was

Fig. 2. Digestion of DNA from mice 73 and 9.02 with Bam HI (lane 1), Bam HI plus Xba I (lane 2), and Bam HI plus Xho I (lane 3). This experiment was performed because the single bands generated by Xba I and Xho I alone were so large that their mobilities could not be distinguished from each other or from the band produced by undigested DNA. Thus, although these large bands were consistent with integration, they did not rule out the possibility that the plasmid DNA existed as a large independent piece without sites for Xba I or Xho I. Cutting first with Bam HI produced smaller bands whose mobilities were altered to a greater degree by a given change in size. The association of the plasmid with DNA containing Xba I sites was therefore more readily demonstrated when Bam HI digestion was performed prior to Xba I. Clearly Xba I alters the Bam HI pattern in both mice. Although the Xba I digest did not proceed to completion in mouse 73, almost all of the 7.8-kb Bam HI band was converted to 5.6 kb by Xba I. The 18-kb Bam HI band in mouse 9.02 was altered to 15 kb by Xba I. Thus, at least 2.2 kb of genomic DNA was ligated to plasmid sequences in mouse 73, and 3 kb of genomic material was ligated to plasmid DNA of mouse 9.02. As expected, double digests of the positive control (PC) did not alter the Bam HI pattern. Failure of Xho I to alter the mobilities of the Bam HI fragments indicates that the genomic portions of these fragments did not contain Xho I sites. Positive controls in these and all other experiments were formulated by adding purified plasmid to DNA from uninjected mice at a ratio of 1 to  $10^6$ , by weight.

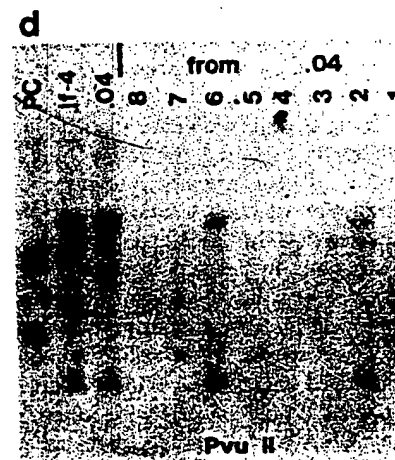
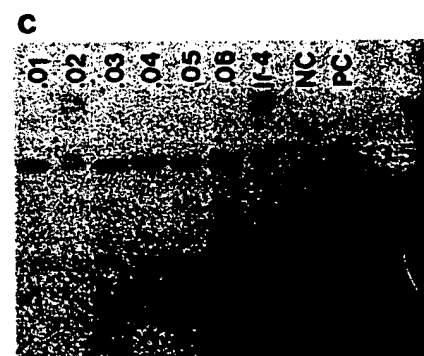
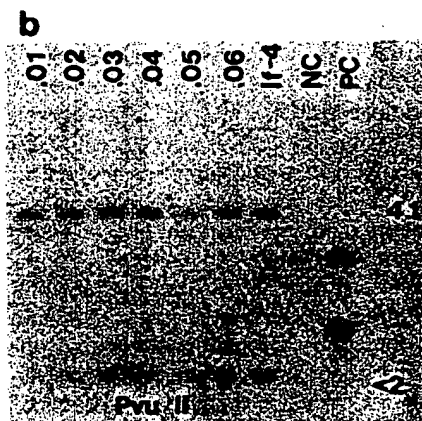
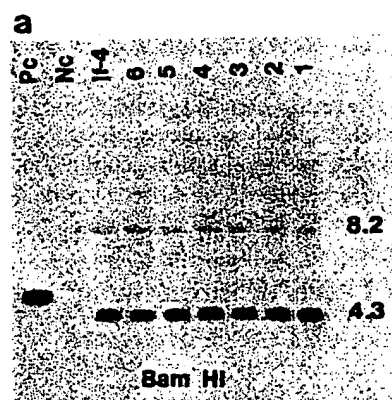
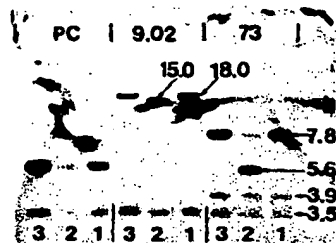


Fig. 3. Spleen DNA from mouse If-4 and six progeny digested (a) with Bam HI, (b) Pvu II, and (c) Xba I, and then probed with plf. Subsequent complete Xba I digests of mouse If-4 and offspring .02 produced the same 13.5-kb band observed in the other samples. These digests demonstrate germ line transmission of plf sequences. (d) Pvu II digest of spleen DNA from mouse If-4, its offspring mouse .04 and eight progeny of .04. Of these eight second-generation progeny mice, 2 and 6 show inheritance of plf sequences; PC indicates the positive control; NC a negative control.

crossed to an uninjected male. Nine of 15 progeny from three litters thus far tested have inherited the pIf-derived sequences. The numbers of offspring with these sequences in each litter were six of six, zero of four, and three of five. The second litter was killed at birth and therefore the sex of the litter members was not examined; however, the sex ratios of the first and third litters were normal (three males out of six, and two males out of five). That all of the first six mice showed the sequence and all of the next four did not was unexpected, but is best explained as a statistical anomaly. The ontogenic history of the mouse primordial germ cell is such that randomization of this cell population occurs prior to entry into genital ridge. At present, there is no evidence that suggests a special relation between oocytes ovulated during any particular estrous cycle.

Consistent with the notion that the sequences were inherited is the observation that the restriction patterns of the DNA from the offspring were indistinguishable from those of the parent. Digestion with Bam HI, Pvu II, or Xba I gave identical patterns in parent and offspring (Fig. 3, a to c). Particularly persuasive is the digest with Xba I; a partial digest of one of the offspring's DNA and of mouse If-4 gave the same multiple bands (Fig. 3c). This result shows that not only are the closest Xba I sites in parent and offspring located at similar distances from the plasmid sequences, but more distant sites are also similarly or identically spaced. Subsequent complete Xba I digests of If-4 and offspring No. 2 resulted in a single band of the same size as the other five offspring (data not shown). These results provide evidence that the pIf sequences were integrated into a host chromosome.

The introduction of foreign DNA in a mouse chromosome without disruption of the meiotic process presents the possibility of producing large colonies of mice carrying transferred sequences. This capability is essential for many kinds of studies of gene transfer into mice. The production of such a colony, however, requires that the transferred material remain stable in the genome over several generations. We tested the stability of the pIf-derived sequences in the If-4 line by breeding one of its offspring to an uninjected male mouse to produce F<sub>2</sub> progeny. Whole animals were killed; and their DNA was extracted, digested with Pvu II, and subjected to filter hybridization with pIf as the probe. Two of the first eight offspring produced by one of the F<sub>1</sub> mice showed clear homology to the probe, with a restriction pattern in-

distinguishable from the F<sub>1</sub> parent or from the original transformed mouse, If-4 (Fig. 3d). This second generation of germ line transmission constitutes evidence for the stability of the transferred material.

The integration of plasmid sequences and their transmission to offspring means, for example, that mice can be backcrossed to produce homozygotes for the transferred sequences, making possible the study of crossover events within a DNA segment whose sequence is well defined, and facilitating mapping studies by both Mendelian and somatic cell genetic approaches. Sequences present in small organs can be studied by pooling tissue from many animals. Breeding tests can also be used to determine whether genes transferred into mice are integrated randomly or reproducibly into a specific site. This issue is of importance if attempts at gene replacement are to be made.

Our data, as well as those from several other laboratories, indicate promise for the technique of pronuclear injection for studying gene action during mammalian development. Our initial report that such injections could succeed in transferring genes into developing mice has been confirmed (11-14). The successful transfer of human insulin into fetal mice by pronuclear injection has been demonstrated (11); and subsequently, the retention of the human  $\beta$ -globin gene in the

DNA of fetal mice was described (12). Supporting evidence for germ line transmission of transferred genes has also been gathered (13, 14), and expression of genes injected into the pronucleus has been observed at late fetal stages and in adult mice (12, 14).

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30 September 1981; revised 30 October 1981

## High Levels of Intracellular Bombesin Characterize Human Small-Cell Lung Carcinoma

**Abstract.** "Small cells" or "oat cells" characterize a virulent form of lung cancer and share many biochemical properties with peptide-secreting neurones. The neuropeptide bombesin is present in all small-cell lines examined, but not in other lung cancer cell lines, suggesting that bombesinergic precursor cells in lung may give rise to this disease.

Approximately 25 percent of all lung cancers are small-cell (oat cell) carcinomas (SCCL), a clinicopathological entity, distinguished from other "non-small-cell" lung cancer histologic types (epidermoid, adenocarcinoma, and large-cell carcinoma) by its characteristic morphology, tendency to metastasize early and widely, frequency of ectopic hormone secretion, and responsiveness to chemotherapy and radiotherapy (1). Well-characterized, clonable SCCL tissue culture lines have greatly advanced our knowledge of the biology of SCCL (2). These SCCL lines are distinguished from those of the other lung cancer types by the presence of neurosecretory gran-

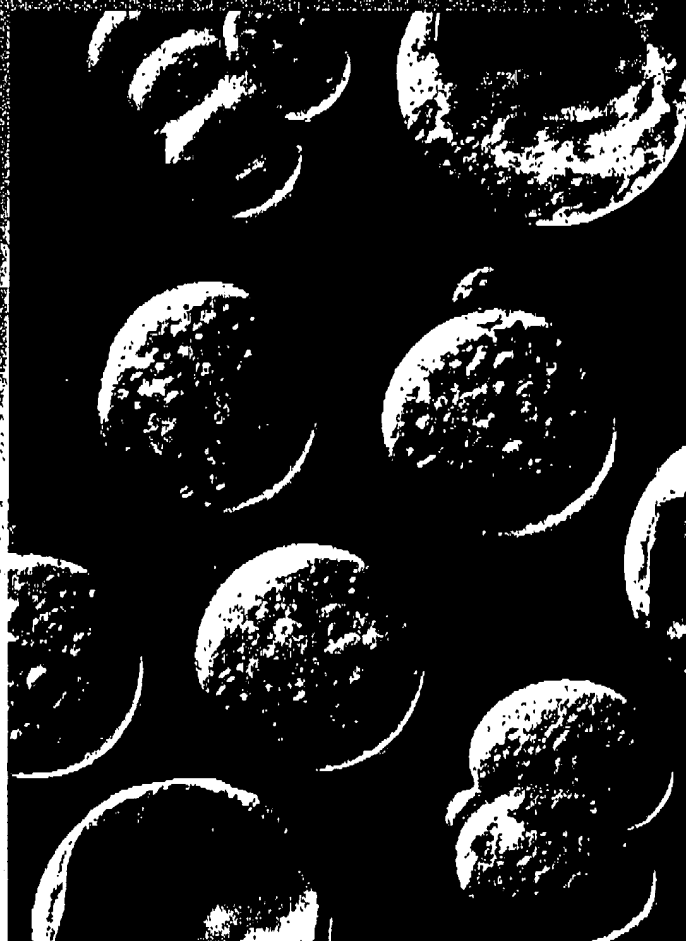
ules, frequent polypeptide hormone secretion, high levels of L-dopa decarboxylase, high levels of the isoenzyme of creatine kinase found in brain, and neuron-specific enolase, as well as a lack of substrate adhesion and characteristic growth factor requirements (2-4). Amine precursor uptake and decarboxylating (APUD) cells consist of a widely distributed network of neuroendocrine cells programmed to secrete certain amines and polypeptide hormones (5); SCCL and the more benign pulmonary carcinoids are presumed to arise from normal APUD cells in the respiratory tract (6). We now report that the 17 SCCL culture lines tested have high quantities of intra-



# TRANSGENIC ANIMAL TECHNOLOGY

Edited by

CARL A. PINKERT



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SECOND EDITION

# Transgenic Animal Technology

**A Laboratory Handbook**

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Center for Aging and Developmental Biology  
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## CHAPTER 1

# Introduction to Transgenic Animal Technology

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- I. Introduction
- II. Historical Background
- III. Applications and Overview of Text
- References

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### I. INTRODUCTION

The last three decades witnessed a rapid advance of the application of genetic engineering techniques for increasingly complex organisms, from single-cell microbial and eukaryotic culture systems to multicellular whole-animal systems. The whole animal is generally recognized as an essential tool for biomedical and biological research, as well as for pharmaceutical development and toxicological/safety screening technologies. Moreover, an understanding of the developmental and tissue-specific regulation of gene expression is achieved only through *in vivo* whole-animal studies.

Today, transgenic animals embody one of the most potent and exciting research tools in the biological sciences. Transgenic animals represent unique models that are custom tailored to address specific biological questions. Hence, the ability to introduce functional genes into animals provides a very powerful tool for dissecting complex biological processes and systems. Gene transfer is of particular value in those animal species, where long life cycles reduce the value of classical breeding practices for rapid genetic modification. For identification of interesting new models, genetic screening and characterization of chance

mutations remains a long and arduous task. Furthermore, classical genetic monitoring cannot engineer a specific genetic trait in a directed fashion.

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## II. HISTORICAL BACKGROUND

In the early 1980s, only a handful of laboratories possessed the technology necessary to produce transgenic animals. With this in mind, this text is envisioned as a bridge to the development of various transgenic animal models. The gene transfer technology that is currently utilized in laboratory and domestic animals was pioneered using the mouse model. Today, the mouse continues to serve as a starting point for implementing gene transfer procedures and is the standard for optimizing experimental efficiencies for other species. Inherent species differences are frequently discounted by researchers who are planning studies with a more applicable species model. However, when one attempts to compare experimental results generated in mice to those obtained in other species, not surprisingly, many differences become readily apparent. Therefore, an objective of this text will be to address the adaptation of relevant protocols.

When initiating work related to gene transfer, it is important to look at the rapid advancement of a technology that is still primitive by many standards. From an historical perspective, one readily contemplates potential technologies and methods that lie just ahead. Whereas modern recombinant DNA techniques are of primary importance, the techniques of early mammalian embryologists were crucial to the development of gene transfer technology. While we can look at just over two decades of transgenic animal production, the preliminary experiments leading to this text go back millennia to the first efforts to artificially regulate or synchronize embryo development. More recently, we observed the centennial of the first successful embryo transfer experiments, dating back to the efforts first published in the 1880s and to Hcape's success in 1891. By the time the studies by Hammond were reported in the late 1940s, culture systems were developed that sustained ova through several cleavage divisions (see Hammond, 1949). Such methods provided a means to systematically investigate and develop procedures for a variety of egg manipulations. These early studies led to experiments that ranged from mixing of mouse embryos and production of chimeric animals, to the transfer of inner cell mass cells and teratocarcinoma cells, to nuclear transfer and the first injections of nucleic acids into developing ova. Without the ability to culture or maintain ova *in vitro*, such manipulations or the requisite insights would not be possible (see Brinster and Palmiter, 1986).

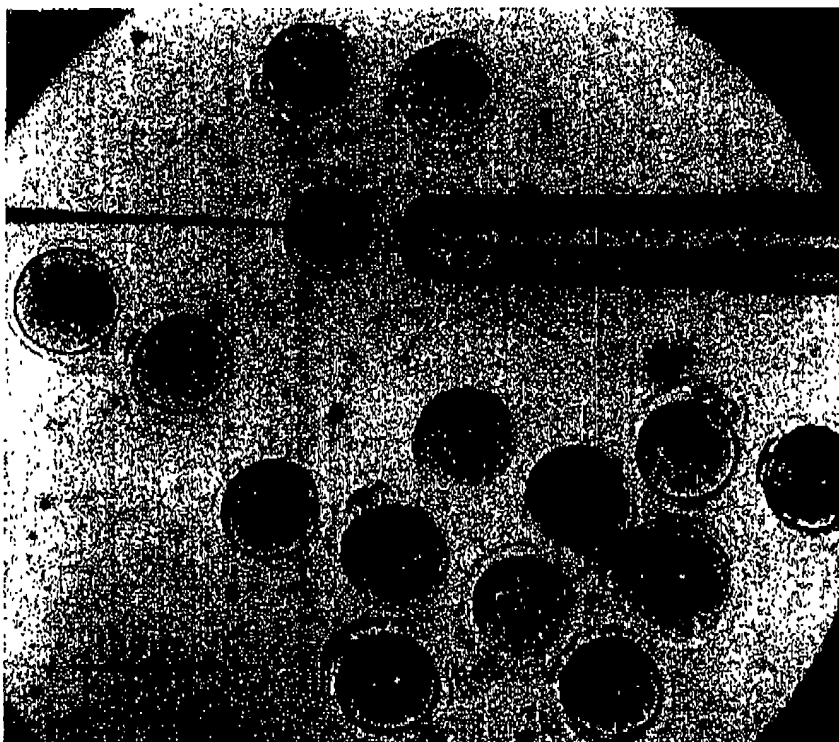
In 1977, Gurdon transferred mRNA and DNA into *Xenopus* eggs and observed that the transferred nucleic acids could function in an appropriate manner. This was followed by a report by Brinster *et al.* (1980) of similar studies in a mammalian system, using fertilized mouse ova in initial experiments. Here, using rabbit globin mRNA, an appropriate translational product was obtained.

## 1. Introduction to Transgenic Animal Technology

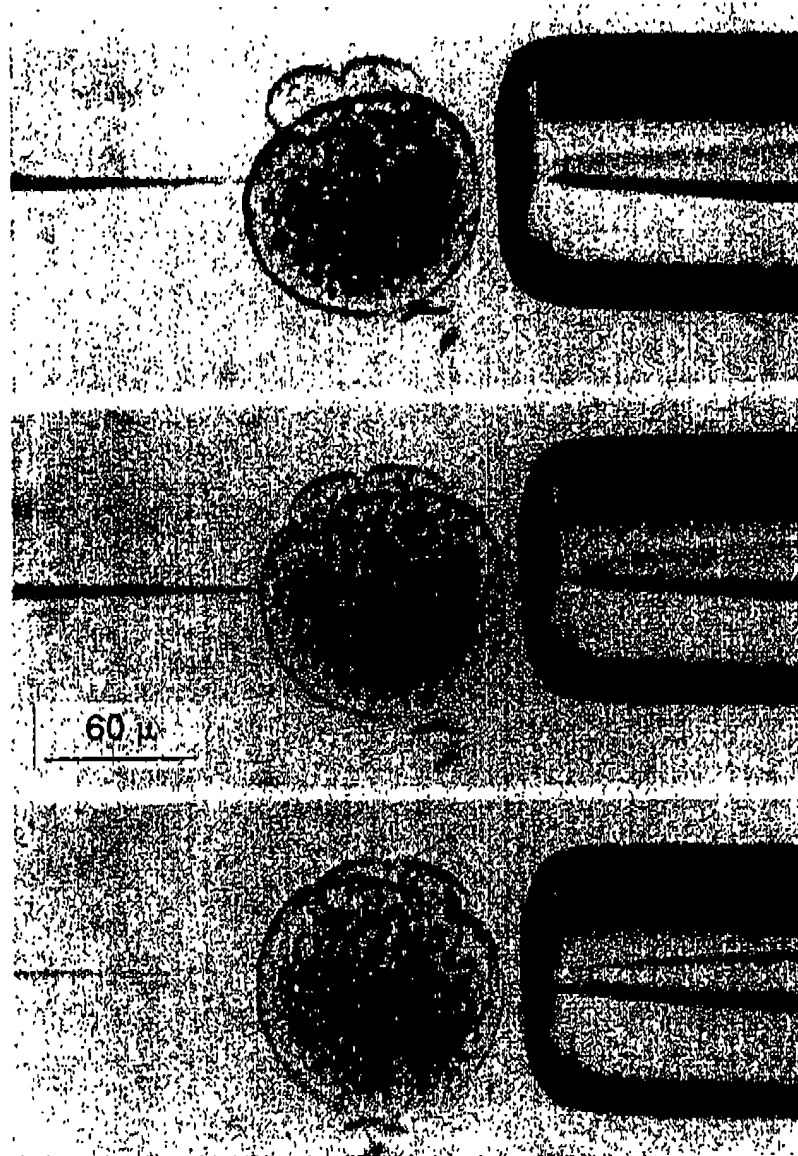
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Major turning points in science continue to accelerate at an incredible pace. The technology available in 1994 has developed considerably and, as predicted, a number of areas do appear antiquated today. It is amazing to look back at the major events related to genetic engineering of animals and how our ability to manipulate the genome has come so far.

The production of transgenic mice has been hailed as a seminal event in the development of animal biotechnology. In reviewing the early events leading to the first genetically engineered mice, it is fascinating to note that the entire procedure for DNA microinjection was described over 35 years ago. While some progress seems extremely rapid, it is still difficult to believe that, following the first published report of a microinjection method in 1966 (Lin, 1966; see Figs. 1 and 2), it would be 15 years before transgenic animals were created. The five pioneering laboratories that reported success at gene transfer (Gordon *et al.*, 1980;



**FIGURE 1** Microinjection of murine zygotes. The initial procedures for DNA microinjection were outlined in 1966. Here, zygotes are being injected with oil droplets. The zygotes survived this mechanical trauma, from use of holding pipettes to insertion of an injection pipette. (Reprinted with permission from Lin, 1966.)



**FIGURE 2** Microinjection of murine zygotes. As described in the 1966 paper by Lin, zygotes survived not only the mechanical trauma associated with the rudimentary injection procedures but the injection of a bovine  $\gamma$ -globulin solution as well. (Reprinted with permission from Lin, 1966.)

Wagner *et al.*, 1981a,b; Harbers *et al.*, 1981; Brinster *et al.*, 1981; Costantini and Lacy, 1981; Gordon and Ruddle, 1981) would not have been able to do so were it not for the recombinant DNA technologies necessary to develop protocols or document results. In gene transfer, animals carrying new genes (integrating foreign DNA segments into their genome) are referred to as "transgenic," a term first coined by Gordon and Ruddle (1981). As such, transgenic animals were recognized as specific variants of species following the introduction and/or integration of a new gene or genes into the genome. As for many technologies, the definition of transgenic animals has taken on a broader meaning and perspective that is more inclusive and includes animals either integrating foreign DNA segments into their genome following gene transfer or resulting from the molecular manipulation of endogenous genomic DNA. (Pinkert *et al.*, 1995). Yet, as outlined by Beardmore (1997), this definition, too, is likely to be refined as we move forward.

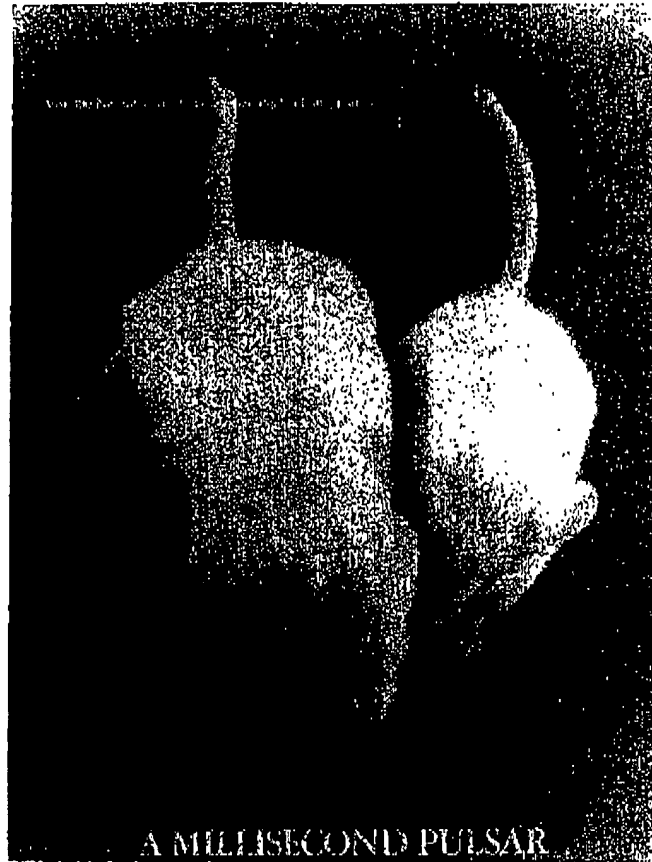
There are now hundreds of excellent reviews that detail the production of transgenic animals, in addition to a journal, *Transgenic Research*, which is dedicated to this field. In the first edition, readers were referred to now classical reviews by Brinster and Palmiter (1986), Bürki (1986), Camper (1987), Cordaro (1989), First and Haseltine (1991), Grosveld and Kollian (1992), Hogan *et al.* (1986), Palmiter and Brinster (1986), Pattengale *et al.* (1989), Pinkert (1987), Pinkert *et al.* (1990), Pursel *et al.* (1989), Rusconi (1991), Scangos and Bieberich (1987), and Van Brunt (1988)]. However, to me, the singular effort with the greatest influence on propelling this technology would not be among the initial reports just described. Rather, early work of Richard Palmiter and Ralph Brinster related to growth performance and the dramatic phenotype of growth hormone transgenic mice (Fig. 3) subsequently influenced the emerging field in a most compelling manner for both basic and applied sciences (Palmiter *et al.*, 1982, 1983).

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### III. APPLICATIONS AND OVERVIEW OF TEXT

Scientists have envisioned many potential studies and applications, if an animal genome could be readily modified. Therefore, the realization of the many technologies at hand today has opened new avenues of research promise. Production of transgenic mice marked the convergence of previous advances in the areas of recombinant DNA technology and manipulation and culture of animal cells and embryos. Transgenic mice provide a powerful model to explore the regulation of gene expression as well as the regulation of cellular and physiological processes. The use of transgenic animals in biomedical, agricultural, biological, and biotechnological arenas requires the ability to target gene expression and to control the timing and level of expression of specific genes. Experimental designs have taken advantage of the ability to direct specific expression (including cell types, tissue, organ type, and a multiplicity of internal targets) and ubiquitous, whole-body expression *in vivo*.





**FIGURE 3** Production of transgenic mice harboring a growth hormone (GH) fusion construct. Animals harboring the GH transgene and expressing the GH gene product grew at a rate 2- to 4-fold greater than control littermates, reaching a mature weight twice that of controls. This dramatic phenotype led the way for the exponential development of gene transfer technology. (Reprinted with permission from Palmiter *et al.*, 1982.)

From embryology to virology, the applications of transgenic mice provide models in many disciplines and research areas. Examples include the following:

- *Genetic bases of human and animal disease and the design and testing of strategies for therapy.* Many human diseases either do not exist in animals or are developed only by "higher" mammals, making models scarce and expensive. Many times, an animal model does not exist and the rationale for development is limited.

## 1. Introduction to Transgenic Animal Technology

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- *Disease resistance in humans and animals.* From a basic research and ethical standpoint, it is imperative that we develop models for enhancing characteristic well-being.
- *Gene therapy.* Models for growth, immunological, neurological, reproductive, and hematological disorders have been developed. Circumvention and correction of genetic disorders are now possible to address using a variety of experimental methods.
- *Drug and product/testing screening.* Toxicological screening protocols are already in place that utilize transgenic animal systems. For preclinical drug development, from a fundamental research perspective, a whole-animal model for screening is essential to understanding disease etiology, investigating drug pharmacokinetics, and evaluating therapeutic efficacy. A comparable and validated need is crucial to product safety testing as well.
- *Novel product development through "molecular farming."* In domestic animals, biomedical proteins have been targeted to specific organs and body fluids with reasonable production efficiencies. Tissue plasminogen activator (TPA), human factor IX, and human  $\alpha 1$  antitrypsin are a few products produced in transgenic animals that are currently in different stages of validation and commercialization.
- *Production agriculture.* Long term, it may become possible to produce animals with enhanced characteristics that will have profound influences on the food we eat, influences ranging from production efficiency to the inherent safety of our food supply.

The numerous strategies for producing genetically engineered animals extend from mechanistic (e.g., DNA microinjection; embryonic stem cell-, nuclear transfer or retrovirus-mediated transfer) as well as molecular (cloning) techniques. As the chapters of this text unfold, it will be apparent that the technology has extended to a variety of animal species in addition to the mouse, including the production of transgenic rats, rabbits, swine, ruminants (sheep, goats, and cattle), poultry, and fish. Although genetically engineered amphibians, insects, nematodes, lower eukaryotes and prokaryotes, and members of the plant kingdom have been acknowledged in the literature, such models are beyond the scope of this text.

Advances in the understanding of promoter-enhancer sequences and external transcription-regulatory proteins involved in the control of gene expression continue to evolve using different model systems. In the systems explored in this text, gene transfer technology is a proven asset in science as a means of dissecting gene regulation and expression *in vivo*. However, the primary question that is addressed concerns the particular role of a single gene in development or in a given developmental pathway. With this caveat, considerations include the ramifications of gene activity—from intracellular to inter- and extracellular events within a given tissue or cell type milieu. Normally, gene function is influenced by *cis*-acting elements and *trans*-acting factors. For transferred genes, the *cis*- and

*trans*-activators in conjunction with the gene integration/insertion event within the host genome influence regulation of both endogenous and transferred genes. Using genes that code for (or are composed of) reporter proteins (e.g., growth hormone or *lacZ* constructs), analysis of transgenic animals has revealed the importance of those three factors in determining the developmental timing, efficiency, and tissue distribution of gene expression. Additionally, transgenic animals have proved quite useful in unraveling *in vivo* artifacts of other model systems or techniques.

Although gene transfer technology continues to open new and unexplored biological frontiers, it also raises questions concerning regulatory and commercialization issues. It is not within the scope of this text, however, to fully address these issues. Suffice it to say that a number of issues exist and will continue to plague the development of many of the systems described herein. Major aspects of the regulation of this technology will focus on the following issues as we begin the twenty-first century:

- Environmental impact following "release" of transgenic animals
- Public perceptions
- Ethical considerations
- Legislation
- Safety of transgenic foodstuffs
- Patent aspects and product uniformity/economics

Contrary to the early prospects related to mainstreaming of this technology, there are numerous societal challenges regarding potential risks that are still ahead. The potential risks at hand in 1994 are still with us today. They still can be defined by scientific evidence but also in relation to public concern (whether perceived or real). Therefore, the central questions will revolve around the proper safeguards to employ and the development of a coherent and unified regulation of the technology. Can new animal reservoirs of fatal human diseases be created? Can more virulent pathogens be artificially created? What is the environmental impact of the "release" of genetically engineered animals? Do the advantages of a bioengineered product outweigh potential consequences of its use? These are but a few of the questions that researchers cannot ignore and must approach. They are not alone, however, as the many regulatory hurdles that exist today will challenge not only scientists and policymakers, but sociologists, ethicists, and legal scholars as well.

The chapters in this text outline the basic techniques that various laboratories currently use to develop transgenic animals. The methods used to initiate experiments, develop vector systems, maintain animals (and the associated husbandry and experimental needs), and analyze and evaluate animals, with the requisite strategies to enhance experimental efficiency, are described at each step of experimentation. Discussion of all interlaboratory variations for each procedure is not feasible. As the chapter authors have learned, the strategies associated with the production of transgenic animals are quite variable, even between laboratories

## 1. Introduction to Transgenic Animal Technology

that utilize the same systems. Therefore, in some instances, alternatives to published and commonly used techniques are presented. However, most of the techniques for extensions to other systems are unique and timely for new investigators. The overall efficiency of many procedures will vary, as will the cost-benefit ratios, but do not let the mechanics of experimentation outweigh the most important reason that one enters into these studies (unless you are a post-doctoral fellow looking for a niche and eventual job placement), which is the development and characterization of a biological model with specific utility.

Our goal is to illustrate a number of variations or novel methods which differ from the standard protocols outlined in detail for the mouse (some of the earlier references on embryology and micromanipulation of ova are listed in the references and include Rafferty, 1970; Daniel, 1971; Bürki, 1986; and Hogan *et al.*, 1994). The organization of this text is designed in a manner to assist those interested in developing an understanding of the basic species differences in transgenic animal research.

For the novice or new trainee, as well as for the experienced researcher, this text should influence proficiency and ultimately help provide an increase in overall productivity. From those wishing to develop a transgenic animal research program, this text will provide an overview of the requirements needed for development of a comprehensive gene transfer program.

There is only one take-home message to readers beyond the development of a desired biological model. An appreciation for the effort involved in each step of experimentation is most important in order to see a project through, from its design and implementation to the validation of a defined animal model. From a personal standpoint, one cannot discount the equal importance of the many unrelated disciplines, from molecular to whole-animal biology, and the necessary training to ensure the overall success of transgenic animal technology.

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# Differential Regulation of Metallothionein-Thymidine Kinase Fusion Genes in Transgenic Mice and Their Offspring

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## Summary

A fusion plasmid, pMK, containing the mouse metallothionein-I promoter/regulatory region joined to the structural gene of herpesvirus thymidine kinase, was introduced into mice by microinjection into fertilized eggs followed by reinsertion of the eggs into foster mothers. Fifteen percent (10 of 69) of the mice developing from this procedure carried pMK sequences. Seven of these mice expressed high levels of viral thymidine kinase in the liver. This enzyme is inducible by heavy metals, as indicated by assay of thymidine kinase activity following sequential partial hepatectomies with or without cadmium treatment. However, glucocorticoid treatment has been ineffective in all transgenic mice tested. The pMK sequences are extensively methylated at a variety of restriction sites, indicating the existence of a *de novo* methylation enzyme. We have analyzed the inheritance of pMK sequences and their expression in several pedigrees. These fusion genes are inherited as though they were integrated into a single chromosome; however, their expression may be extinguished, diminished or enhanced in the offspring relative to that of the parent. In some animals there is a correlation between changes in DNA methylation and expression of these fusion genes.

## Introduction

DNA has been introduced into animals by microinjection of fertilized eggs and then implantation of these eggs into the reproductive tracts of pseudopregnant females (Gordon et al., 1980; Brinster et al., 1981; Constantini and Lacy, 1981; Harbers et al., 1981; Rusconi and Schaffner, 1981; E. Wagner et al., 1981; T. Wagner et al., 1981). Analyses of the DNA in the adults that develop from these eggs reveal that up to 40% of them may retain injected DNA and that the sequences are often tandemly duplicated. Mice that retain DNA introduced in this manner have been called transgenic mice (Gordon and Ruddle, 1981). In one case, the DNA was shown by *in situ* hybridization to be integrated into one of the mouse chromosomes (Constantini and Lacy, 1981). Consistent with the

chromosomal integration of injected DNA are the observations that it is detected in a variety of tissues examined and that it is passed on through the germ line as expected for chromosomal inheritance. In some cases the acquired genes are also expressed (Brinster et al., 1981; E. Wagner et al., 1981; T. Wagner et al., 1981). This microinjection approach allows access to a number of important developmental problems and provides a means of introducing new genetic traits into animals. Ideally, conditions should be found that optimize both stable integration and expression of injected genes. Longer-range goals include site-specific integration and tissue-specific expression.

To investigate some of these questions, we have constructed a plasmid, pMK, that fuses the promoter/regulatory region of the mouse metallothionein-I (MT-I) gene to the structural gene of herpes simplex virus (HSV) thymidine kinase. This fusion gene is designated MK, and mice that express this gene are called MaK if they are males and MyK if they are females, followed by the number of their foster mother. This gene is particularly advantageous for these studies because part of it is homologous to the endogenous, unique-sequence MT-I genes, thus potentially allowing integration by homologous recombination, while the HSV thymidine kinase part is foreign to the mouse, thereby providing an easy means of detection by hybridization. The HSV thymidine kinase enzyme can be distinguished from endogenous thymidine kinase by a number of methods (Brinster et al., 1981). The MT-I gene is transcriptionally regulated by heavy metals and glucocorticoid hormones both *in vivo* and in a variety of cell lines (Durnam and Palmiter, 1981; Hager and Palmiter, 1981; Mayo and Palmiter, 1981); thus we expected that this fusion gene might be regulated similarly. Indeed, we have shown that expression of the MK gene is regulated by cadmium when it is either transfected into mouse L cells (Mayo et al., 1982) or injected into mouse eggs (Brinster et al., 1982). Deletion mapping reveals that the minimal DNA sequence required for heavy metal regulation lies within 90 bp of the transcription start site (Brinster et al., 1982). Although MT-I mRNA is present and inducible in most mouse tissues, the amount of MT-I mRNA varies considerably from tissue to tissue (Durnam and Palmiter, 1981). These tissue-specific differences in MT-I gene expression provide a rich background against which the expression of acquired MK genes can be compared.

Previously we showed that one transgenic mouse resulting from the injection of pMK had a high level of HSV thymidine kinase activity (Brinster et al., 1981). We have analyzed six more transgenic mice that expressed HSV thymidine kinase, and have focused on the following questions: Is the frequency of obtaining mice that express HSV thymidine kinase correlated with the DNA construction injected? Are the MK genes

regulated by heavy metals or glucocorticoids? Does HSV thymidine kinase activity correlate with MK-gene copy number? Are MK genes transmitted to progeny, and do the progeny express these genes in the same way as their parent? If not, does HSV thymidine kinase expression in the offspring correlate with loss or rearrangement of MK sequences? Does injected DNA become methylated? If so, are specific sequences methylated in a consistent manner from animal to animal; does the methylation pattern correlate with MK gene expression; and is it stable in subsequent generations?

## Results

### Expression of HSV Thymidine Kinase Activity

Table 1 summarizes the results obtained from the seven mice that expressed HSV thymidine kinase activity out of 69 mice that developed from eggs injected with pMK. Four different DNA constructions were tried: circular plasmid pMK; pMK linearized with Bam HI; Bam HI-cut pMK ligated with a sixfold weight excess of Bam HI-cut mouse DNA; and a blunt-ended, linear 2.1 kb Bst EII fragment containing the MK gene (see Figure 1). In anticipation of heavy metal regulation of MK gene expression, the mice were injected with CdSO<sub>4</sub> 18 hr prior to partial hepatectomy and thymidine kinase assay. Thymidine kinase activity is

expressed both in absolute terms and as the ratio of activities without and with an antibody specific for HSV thymidine kinase (Brinster et al., 1981). Although the parameters generally parallel each other, it is important to measure both because the endogenous thymidine kinase activity in the liver declines dramatically after birth, and the mice were assayed at different ages. Table 1 shows that 7 out of 10 mice that retained pMK sequences expressed viral thymidine kinase with activities that ranged from 4 to 70 times the endogenous thymidine kinase activity. The endogenous thymidine kinase activity of 62 mice assayed at comparable ages ranged from 0.5 to 1.5 cpm/ $\mu$ g/min, and the activity ratio with and without antibody ranged from 0.7 to 1.7. There was no obvious relation of HSV thymidine kinase activity to either the number of DNA copies present (see below) or the DNA construction injected.

### Regulation of HSV Thymidine Kinase Activity

To ask whether MK genes can be regulated in adult mouse liver, we surgically removed samples of liver following injection of either CdSO<sub>4</sub> or dexamethasone; controls were untreated. Serial hepatectomies were separated by about a month. The results (Table 2) indicate that cadmium induced HSV thymidine kinase activity 10 to 50 fold in four transgenic mice, whereas glucocorticoids were ineffective. In some experi-

Table 1. Summary of Transgenic Mice That Expressed HSV Thymidine Kinase

	MaK-23	MaK-116	MyK-67	MaK-67	MyK-84	MyK-103	MaK-113	Total
No. of MK genes per cell <sup>a</sup>	8	2	2	150	100	1	32	
DNA construction <sup>b</sup>	A	A	C	C	D	B	B	
No. of mice that expressed HSV thymidine kinase	2		2		1	2		7
No. of mice carrying pMK sequences	5		2		1	2		10
Total no. of mice analyzed	14		35		5	15		69
Thymidine kinase activity cpm/ $\mu$ g/min	122	10.8	68	74	62	18.5	5.5	
-Ab/+Ab ratio <sup>c</sup>	30	7.2	11	71	17	10.0	4.2	
No. of offspring that expressed HSV thymidine kinase	0 <sup>d</sup>	3 <sup>e</sup>	0 <sup>f</sup>	2 <sup>g</sup>	12 <sup>h</sup>	3 <sup>i</sup>	1 <sup>j</sup>	
No. of offspring that carried pMK sequences	0 <sup>d</sup>	3	4	34	14	3	2	
Total no. of offspring analyzed	0	11	9	54	32	35	10	

<sup>a</sup> Gene copy number was estimated from dot blots (as described in Figure 2) or restriction digests.

<sup>b</sup> A: supercoiled pMK. B: pMK linearized with Bam HI. C: pMK linearized with Bam HI and ligated with a sixfold excess of Bam-cut mouse DNA. D: linear 2.1 kb Bst EII fragment, blunt-ended with Klenow fragment of DNA polymerase I (see Figure 1).

<sup>c</sup> Total thymidine kinase activity divided by the residual activity remaining after addition of excess antisera to HSV thymidine kinase (see Brinster et al., 1981). For pMK-negative mice this ratio ranges from 0.7 to 1.7 (n = 62).

<sup>d</sup> No offspring.

<sup>e</sup> The HSV thymidine kinase activities of the three offspring positive for MK sequences ranged from 49 to 79 cpm/ $\mu$ g/min.

<sup>f</sup> All of the offspring died prior to thymidine kinase assay.

<sup>g</sup> Only 13 of the 34 mice positive for MK sequences have been analyzed for thymidine kinase activity (see Figure 3).

<sup>h</sup> Only 12 of the 14 mice that carried MK sequences have been analyzed; their thymidine kinase activities ranged from 13 to 451 cpm/ $\mu$ g/min. Thymidine kinase activity has been retained in the second generation of MyK-84-7 as well.

<sup>i</sup> The thymidine kinase activities of the three offspring positive for MK sequences ranged from 10 to 106 cpm/ $\mu$ g/min.

<sup>j</sup> Only one of the two mice positive for MK sequences has been analyzed; its thymidine kinase activity was the same as that of the mother.

**Table 2. Regulation of HSV Thymidine Kinase Activity**

Mouse	Hepatectomy	Treatment	Thymidine Kinase Activity (cpm/ $\mu$ g/min) <sup>a</sup>	
			-Ab	+Ab
MaK-67	1st	Cadmium <sup>b</sup>	76.5	1.1
	2nd	None	3.3	1.5
	3rd	Dexamethasone <sup>b</sup>	3.1	2.2
MyK-67	1st	Cadmium <sup>b</sup>	68.3	6.2
	2nd	Dexamethasone <sup>c</sup>	1.5	1.1
	3rd	Cadmium <sup>b</sup>	55.0	1.5
MyK-84-9	1st	Dexamethasone <sup>c</sup>	3.0	1.1
	2nd	Cadmium <sup>c</sup>	28.0	1.3
	3rd	None	3.3	1.7
MyK-103	1st	Cadmium <sup>b</sup>	18.5	1.8
	2nd	Dexamethasone <sup>c</sup>	1.2	0.5

<sup>a</sup> Measured as described by Brinster et al. (1981). Ab: antibody specific for HSV thymidine kinase.

<sup>b</sup> CdSO<sub>4</sub> (1 mg/kg) or dexamethasone (0.5 mg/kg) was administered 18 hr before hepatectomy.

<sup>c</sup> CdSO<sub>4</sub> (1 mg/kg) or dexamethasone (0.5 mg/kg) was administered 18 and 4 hr before hepatectomy.

ments, two injections of dexamethasone were given, and endogenous MT-I mRNA was measured to ascertain whether the regimen was effective. Under these conditions MT-I mRNA was induced to a level of 1200 molecules per cell, indicative of optimal treatment (Hager and Palmiter, 1981).

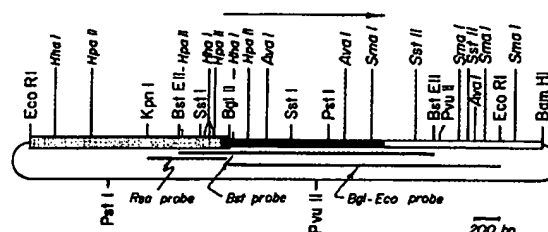
### Analysis of MK-Gene Copy Number

Quantitation of DNA copies was accomplished by a dilution dot hybridization procedure. DNA from MK-positive mice was serially diluted into normal mouse DNA, and 10  $\mu$ g was spotted onto nitrocellulose, baked and then hybridized with the Bgl-Eco probe that spans the thymidine kinase structural gene (Figure 1). Figure 2 illustrates the technique with the seven mice listed in Table 1 and two of their offspring. Analysis of Southern blots suggests that MyK-103 has a single copy of pMK DNA. By comparison with MyK-103, we estimate that the DNA copy number in mice expressing MK genes ranges from 1 to 150, as indicated in Table 1.

Because of the sensitivity of this simple dot hybridization procedure, we have now modified our general strategy such that the presence of pMK DNA can be analyzed without complicated surgery. We extract nucleic acids from the terminal quarter of the tail and perform a "tail blot" as described in the Experimental Procedures. By including appropriate standards, we can readily discern a single insert. Animals that are positive for pMK are then analyzed for MK gene expression.

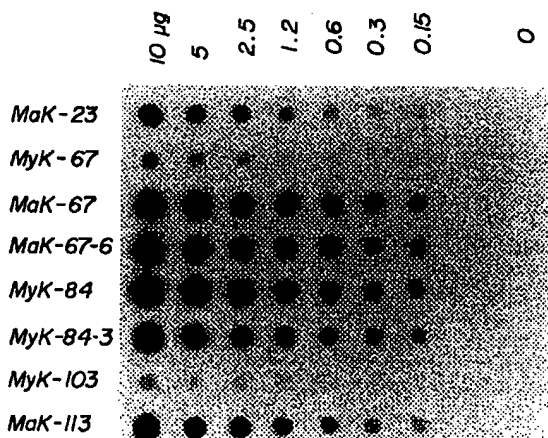
### Expression of MK Genes in Offspring of MaK-67

Fifty-four offspring of MaK-67 have been analyzed for the presence of pMK DNA. Thirty-four carried the



**Figure 1. Partial Restriction Map of Plasmid pMK**

This 8.4 kb plasmid was constructed by fusion of the mouse MT-I gene to the HSV thymidine kinase gene at their unique Bgl II sites (see Brinster et al., 1981). Wide box: the MT-I region. Thin box: the HSV thymidine kinase region. Thin line: pBR322. Solid boxes: region that is expressed as mRNA. Arrow: the direction of transcription. The position of the restriction enzyme sites examined in this study are indicated. Italics: those that are sensitive to cytidine methylation. The pBR322 region is not drawn to scale. Only those Hha I and Hpa II sites that would be detected by the Rsa probe are shown; there are many more of these sites in the HSV thymidine kinase region (M. Wagner et al., 1981). DNA fragments corresponding to the three probes were isolated from agarose gels and nick-translated to high specific activity as described in the Experimental Procedures.



**Figure 2. Dot Hybridization of DNA from Transgenic Mice**

The indicated amounts of DNA were spotted onto nitrocellulose, baked and hybridized with the Bgl-Eco probe as described in the Experimental Procedures. MaK-67-6 and MyK-84-3 are offspring.

same amount of pMK sequences as the father (as measured by dot hybridization), while 20 carried none. The pedigree and HSV thymidine kinase activity of the first 19 offspring are shown in Figure 3. Remarkably, the expression of pMK genes is variable: of 13 offspring carrying MK sequences, one had moderately high activity, one had slight activity and 11 had no significant activity. A second hepatectomy was performed on four of the nonexpressers after a fivefold higher dose of CdSO<sub>4</sub>. Again, the activity ratios revealed no significant HSV thymidine kinase activity (Figure 3), even though MT-I mRNA levels were induced to maximal levels (3500–7300 molecules per cell). To determine whether MK genes would be transmitted through the female germ line as well, and to ask whether MK gene expression might be reacti-



vated, we analyzed a second generation of offspring of two nonexpressing females, MaK-67-9 and MaK-67-11. The MK genes segregated to half of the offspring (9 of 19), but none of these offspring displayed significant HSV thymidine kinase activity (Figure 3).

#### Analysis of pMK DNA in MaK-67 and His Offspring

A simple explanation for the variable expression of MK genes in the offspring might be that the offspring did not receive identical copies of the MK gene, perhaps as a result of integration into different chromosomes of the father or because of rearrangement during meiosis or development of the offspring. Figure 4 suggests that neither of these explanations is correct, because the restriction pattern of the offspring that expressed HSV thymidine kinase (MaK-67-6 and MaK-67-8) was indistinguishable from that of offspring that did not express HSV thymidine kinase. Furthermore, there was no obvious difference in the restric-

tion pattern between different tissues (liver and kidney) of the father and the offspring. MaK-67 developed from an egg injected with pMK that had been linearized with Bam HI and ligated to a sixfold excess of Bam HI-cleaved mouse DNA. This construction, coupled with the presence of many copies of pMK (Figure 2), explains the very complicated restriction patterns obtained (Figure 4). The band hybridizing most intensely in the Bgl II digest represents unit-length plasmid presumably derived from tandem repeats of pMK. Bst EII, which cuts twice within pMK, generates two intense bands representing fragments predicted from unintegrated or tandem repeats of pMK. The other bands may represent junction fragments of pMK with mouse DNA that was ligated either in vitro or at the site(s) of integration.

We also examined the inheritance of pMK sequences by the second generation. Offspring of females MaK-67-9 and MaK-67-11 had the same Bgl II restriction pattern as their mothers (Figure 4, inset). The segregation of MK genes in the first generation was skewed significantly (34 of 54), while in the second generation the segregation was 9 of 19. The observation that MK gene dose and restriction pattern are the same in all offspring that receive any MK genes indicates that the MK genes are probably integrated into a single chromosome.

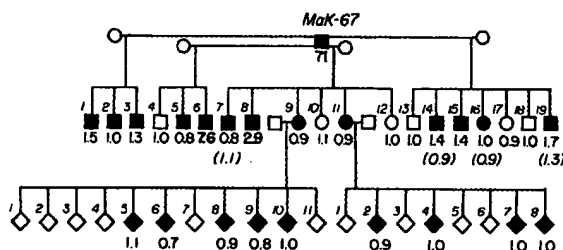


Figure 3. Pedigree of MaK-67

Solid symbols: distribution of MK gene sequences. Mice were injected with 1 mg/kg CdSO<sub>4</sub> 18 hr before partial hepatectomy. Thymidine kinase activity ratios (without antisera against HSV thymidine kinase/with antisera) are given under the symbols; four of the first-generation offspring were also assayed 18 hr after injection of 5 mg/kg body weight CdSO<sub>4</sub> (these thymidine kinase activity ratios are shown in parentheses).

#### Methylation of pMK DNA in MaK-67 and His Offspring

We next asked whether pMK sequences become methylated, and if there are changes in the methylation pattern that correlate with expression. Figure 5 shows that both the Sma I (CCCGGG) sites and Sst II (CCGCGG) sites that lie within the Bgl-Eco probe are extensively methylated, as indicated by the fact that

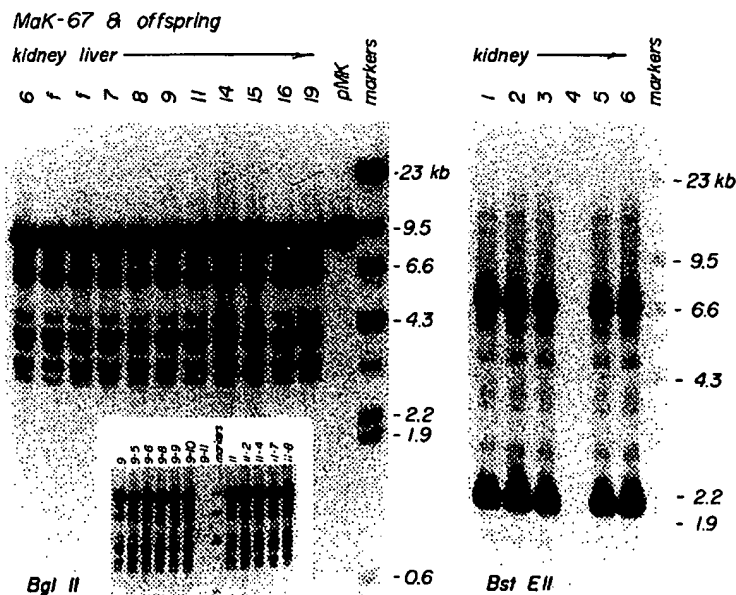


Figure 4. Restriction Analysis of DNA from MaK-67 and His Offspring

Kidney or liver DNA (5 µg) was digested with Bgl II (left) or Bst EII (right), subjected to electrophoresis on a 0.7% agarose gel, transferred to nitrocellulose and hybridized with a probe that includes the entire plasmid except the 1 kb Eco RI-Kpn I region shown in Figure 1. (Lanes 1) DNA from father; (numbered lanes) DNA from corresponding offspring. (Lane pMK) 100 pg plasmid DNA. (Inset) Bgl II restriction pattern of the second generation—that is, offspring of MaK-67-9 and MaK-67-11 (see Figure 3). The father and offspring MaK-67-8 and MaK-67-8 expressed HSV thymidine kinase (see Figure 3). (Lane markers) λ DNA cut with Hind III and end-labeled.

in double digests with Eco RI and either Sma I or Sst II the major hybridizing band corresponded to the 4.0 kb band generated by Eco RI alone. Controls showing the digestion of pMK by Sst II and Sma I were also carried out. The methylation pattern was identical in liver and kidney DNA of MaK-67. We know that the sequences essential for cadmium regulation of MT-I gene expression lie within one hundred bases of the transcription start site (Brinster et al., 1982; Mayo et al., 1982); thus it seems plausible that methylation of specific residues in this region may affect MK gene expression. A probe that spans this region was used in the experiment shown in Figure 6. This probe also detects endogenous MT-I genes; hence controls showing the pattern obtained with DNA from a pMK-negative offspring, and the same DNA supplemented with the original pMK plasmid, were included to reveal the endogenous bands and to control for complete digestion. Figure 6 shows that most of the Hpa II sites (CCGG) and Hha I sites (GCGC) are methylated, because the bulk of the hybridizing DNA is larger than 10 kb and there are over 30 Hpa II or Hha I sites within pMK. We do note one low molecular weight band in the Hha I digest that appeared when DNA from offspring MaK-67-6 (expressing) was compared with offspring MaK-67-5 (nonexpressing).

#### Analysis of the Restriction and Methylation Patterns of DNA from MyK-84 and Her Offspring

MyK-84 developed from an egg injected with about 400 copies of a blunt-ended, 2.1 kb Bst EII fragment that included the entire MK gene (see Figure 1). Figure

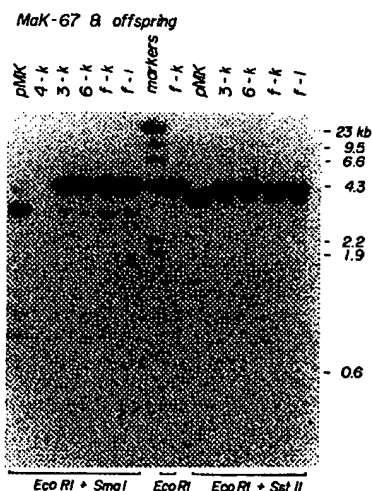


Figure 5. Analysis of Methylation at Sma I and Sst II Sites of MaK-67 and Offspring

Liver (-l) or kidney (-k) DNA (5 µg) from MaK-67 (f) and his offspring (numbers) was digested with Eco RI and either Sma I or Sst II as indicated. The digests were subjected to electrophoresis on a 1.6% agarose gel, transferred to nitrocellulose and hybridized with the Bgl-Eco probe. λ DNA (0.7 µg) was included in each sample to check for complete digestion. (Lanes pMK) Controls in which 20 pg pMK was added to 5 µg of kidney DNA from offspring MaK-67-4.

7 reveals that five of the first ten offspring of MyK-84 contained MK DNA, and four of them (the fifth one died prior to activity analysis) expressed HSV thymidine kinase activity. The HSV thymidine kinase activity of two of the offspring was about threefold greater than that of the mother, while that of the other two was one-half to one-fourth that of the mother; there appears to be a correlation of high activity with the loss of about half of the MK DNA sequences. Figure 8 shows that the hybridizing DNA was of high molecular weight when digested with either Bst EII or Eco RI,

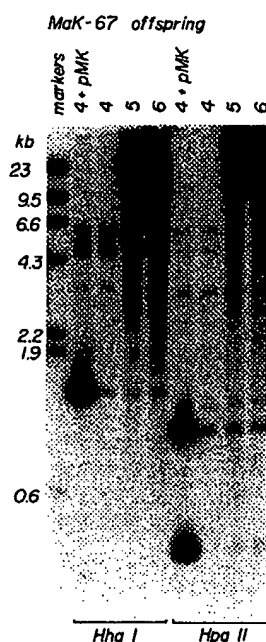


Figure 6. Analysis of Methylation at Hha I and Hpa II sites of MaK-67 Offspring That Expressed (MaK-67-6) and Did Not Express (MaK-67-5) HSV Thymidine Kinase

DNA (10 µg) was digested, subjected to electrophoresis on a 1.2% agarose gel, transferred to nitrocellulose and hybridized with the Rsa probe (see Figure 1). (Lanes 4 + pMK and 4) Controls of MaK-67-4 DNA with and without 150 pg pMK DNA.

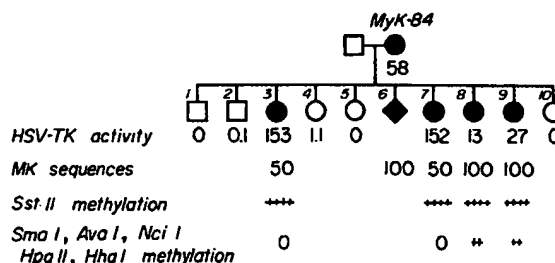


Figure 7. Pedigree of MyK-84

Mice were injected with CdSO<sub>4</sub> (1 mg/kg) 18 hr before partial hepatectomy. Thymidine kinase activity is expressed as cpm/µg/min minus that measured in the presence of antisera specific for HSV thymidine kinase. The number of MK genes was estimated by dot hybridization and from Southern blots. The extent of methylation of specific restriction sites was determined as shown in Figures 9 and 10. ++++: complete methylation. ++: partial methylation. 0: no methylation.

enzymes that do not cut within the original fragment. The absence of digestion with Bst EII confirms the complete destruction of these sites by treatment with DNA polymerase. Enzymes that cut once within the Bst EII fragment (such as Pst I and Bgl II) gave rise to one predominant band of 2.1 kb plus several presumed junction fragments, while enzymes that cut twice (such as Sst I) gave rise to two major bands that

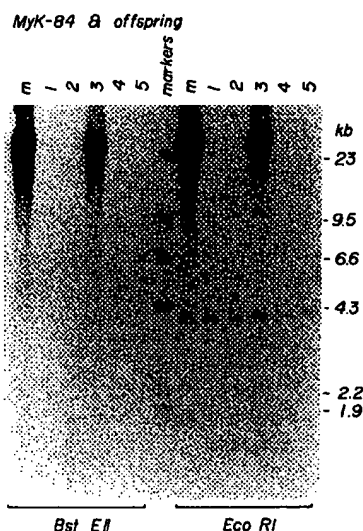


Figure 8. Restriction Analysis of DNA from MyK-84 and Five of Her Offspring

DNA (10 µg) was digested with Bst EII or Eco RI, subjected to electrophoresis on a 1.0% agarose gel, transferred to nitrocellulose and hybridized with the Bst probe shown in Figure 1. DNA of the mother (lane m) and offspring MyK-84-3 (lanes 3) was not cut appreciably by either enzyme, indicating that high molecular weight concatemers of the 2.1 kb Bst EII fragment were generated. The 4 kb hybridizing band in the Eco RI digests represents the MT-I gene. Two faint bands at 5.0 and 5.5 kb were visible in Bst EII digests with longer exposure.

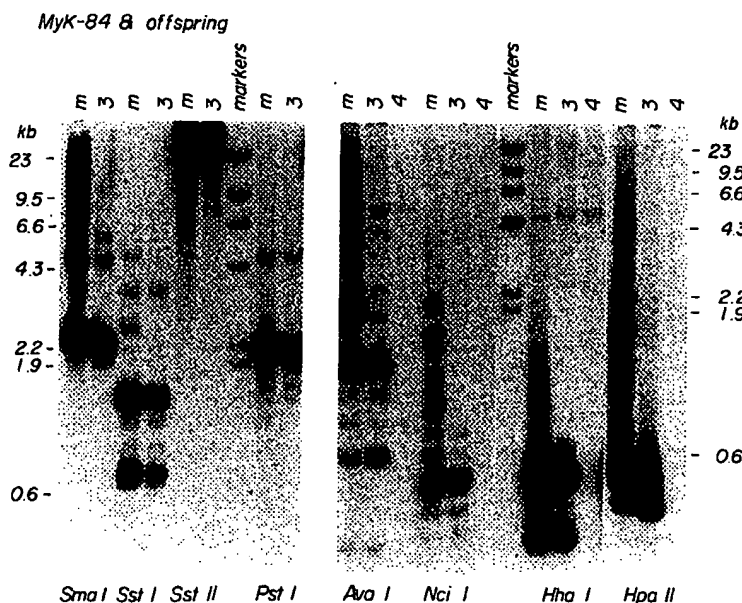


Figure 9. Analysis of Organization and Methylation of MK Genes in MyK-84 and Offspring MyK-84-3

(Lanes m) MyK-84; (lanes 3) MyK-84-3. DNA (10 µg) was digested with the enzymes indicated and subjected to electrophoresis on a 1.0% agarose gel (left) or a 1.5% agarose gel (right). After transfer to nitrocellulose, the DNA was hybridized with the Bgl-Eco probe (left) or Bst probe (right). Because the Bst probe is homologous to a portion of the endogenous MT-I gene, control DNA from offspring MyK-84-4 (lanes 4) is included on the right.

add up to 2.1 kb (Figures 9 and 10). These results are only compatible with a head-to-tail, tandem duplication of the original 2.1 kb fragment, probably integrated into a single chromosome.

The extent of CG methylation in MyK-84 and her offspring was examined with several enzymes (Figures 9 and 10, summarized in Figure 7). Sma I only partially cut the repeated Bst EII fragment in both the mother and offspring MyK-84-8 and MyK-84-9, whereas in the two offspring that expressed high levels of HSV thymidine kinase (MyK-84-3 and MyK-84-7) the Sma I sites were extensively digested. A similar correlation between methylation and HSV thymidine kinase expression was noted in digestions with Ava I, Nci I, Hha I and Hpa II. We were concerned that this might represent partial cleavage in the case of the mother and offspring MyK-84-8 and MyK-84-9, but a repeat digest with twice the amount of enzyme and half the amount of DNA gave exactly the same pattern. Moreover, inclusion of visible amounts of DNA from phage λ in the digests revealed its complete digestion. It is also noteworthy that this disparity was noted only with enzymes that are sensitive to methylation. Thus we favor the idea that methylated copies of the Bst EII fragment were preferentially lost in some offspring when the DNA was transmitted to the next generation. Although most of the CG sequences analyzed were unmethylated in offspring MyK-84-3 and MyK-84-7, the Sst II sites were fully methylated in MyK-84 and all of her offspring; thus loss of methylation is sequence-specific.

#### The Bst EII Fragment Did Not Integrate Into a Homologous DNA Sequence

The Bst EII fragment is the only DNA construction tested so far that does not contain the DNA repeat

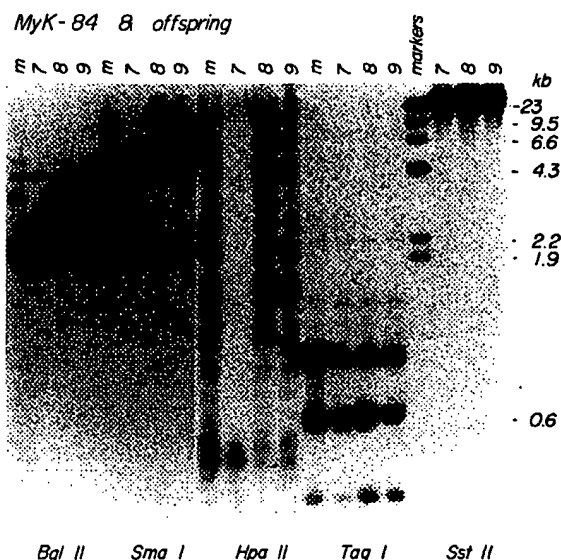


Figure 10. Analysis of Methylation of MK Genes in MyK-84 and Several Offspring

Liver DNA (5  $\mu$ g) was digested with the enzymes indicated, subjected to electrophoresis on 1.5% agarose gels, transferred to nitrocellulose and hybridized with the nick-translated Bgl-Eco probe. (Lanes m) MyK-84; (lanes o) offspring.

sequence located between the Eco RI and Kpn I sites 5' of the MT-I gene (see Figure 1). It contains only 350 bases of unique DNA that are homologous to the 5' end of the MT-I gene and 1750 bases of the HSV thymidine kinase gene. Thus it affords the opportunity to ask whether integration occurs by homologous recombination. The blot shown in Figure 8 reveals a 4.0 kb Eco RI band in all lanes that represents the endogenous MT-I gene. The intensity of this band is essentially the same in all lanes, and no new bands appear, indicating that the Bst EII fragments did not integrate within the 350 bp stretch of homologous sequences present in the mouse genome. We also used a probe specific to the MT-I gene and obtained the same result (data not shown).

## Discussion

### DNA Integration

Several reports indicate the successful introduction of foreign DNA into mice or frogs (see Introduction). The overall efficiency of obtaining stable incorporation of foreign DNA into the mouse genome was 10%–15% in our experiments. We have tried several different constructions (Table 1) in an attempt to improve the efficiency, but the data do not indicate that any of these constructions has a significant effect on the outcome.

We and others have noted that when multiple copies of plasmid or viral DNA are integrated they tend to be tandemly duplicated. In our first experiments, our plas-

mid preparation contained some oligomers, so we could not be certain whether the tandem duplication occurred in the bacterial or mouse cells. The experiment that gave rise to MyK-84 is informative in this regard because the DNA was a blunt-ended Bst EII fragment. Restriction analysis of DNA from MyK-84 revealed a tandem, head-to-tail organization of about 100 copies. A tandem array could occur either by ligation of linear monomers or by circularization followed by homologous recombination or possibly replication of the circles. Only the latter mechanisms readily explain the head-to-tail organization. Consistent with the latter models, we have observed circularization and supercoiling of this particular fragment when it was injected into mouse eggs and analyzed 22 hr later by agarose gel electrophoresis (data not shown). This tandem repeat did not integrate into the mouse genome in the region of extensive homology because the endogenous MT-I genes were intact; however, there may have been small regions of homology elsewhere in the genome that were utilized. The answer to this question may be obtained by the cloning and sequencing of junction fragments. Although we have not shown directly that any of these mice have integrated foreign DNA into one chromosome, this possibility readily accounts for the stable inheritance of the DNA in an all-or-none fashion with a frequency approaching 50% (see Table 1).

### MK Gene Expression

We have detected MK gene expression in seven animals that developed from microinjected eggs out of ten that had pMK sequences. Thus the probability of expressing this gene in the liver is considerably better than we initially observed (Brinster et al., 1981). It is apparent, however, that the HSV thymidine kinase activity of these mice does not correlate with gene dosage (Table 1). The fact that mice with one or two MK genes, for example, MyK-67, may have HSV thymidine kinase activity comparable with that in mice with many copies leads us to suggest that only one or a few copies of the genes are active in mice with many copies.

In most experiments we attempted to induce MK gene expression by administering cadmium to the animal 18 hr before partial hepatectomy. The experiment described in Table 2 shows that this treatment had a dramatic effect on the resulting HSV thymidine kinase activity, inducing it about 50-fold in MaK-67 and MyK-67. This induction is similar to that observed for MT-I mRNA in liver (Durnam and Palmiter, 1981) and some mouse cell lines (Mayo and Palmiter, 1981). The absolute number of MK mRNA molecules that accumulate in the liver of transgenic mice or in transfected L cells after induction with cadmium is 0.1%–1% of the number of MT-I mRNA molecules (Brinster et al., 1981; Mayo et al., 1982). In contrast, transfection of MT-I genes into mouse or human cells

results in accumulation of nearly normal amounts of mRNA (Mayo et al., 1982), suggesting that integration into foreign DNA is not the primary explanation for the low yield of MK mRNA. Perhaps MK genes are transcribed inefficiently or the resulting hybrid mRNA is much more unstable than MT-1 mRNA.

Dexamethasone has not induced HSV thymidine kinase activity in any of the transgenic mice tested so far (Table 2). Although disappointing, this result is consistent with our failure to maintain transcriptional control of the MT-1 gene by glucocorticoids when it is transfected into either human or mouse cells (Mayo et al., 1982) or when it is amplified by selection for cadmium resistance (Mayo and Palmiter, 1982). These observations suggest that some cis-acting modification that is essential for glucocorticoid regulation but not for heavy metal regulation has been lost during gene manipulation.

The finding that MK gene expression is not necessarily stable from one generation to the next is both surprising and perplexing. This phenomenon is illustrated by the analysis of MaK-67 and MyK-84 pedigrees (Figures 3 and 7) as well as those of most of the other transgenic mice (see Table 1, footnotes e-j). We have considered several explanations for these findings. The inherent variability in the thymidine kinase assay is too small to explain order-of-magnitude differences or total extinction of activity. We are confident that cadmium is reaching the liver because MT-1 mRNA levels have been monitored. We have also considered the possibility of DNA loss or rearrangement between generations. The restriction pattern of MK sequences in MaK-67 is very complicated because of the presence of many copies and the particular gene construction that we injected. Nevertheless, it provides an opportunity to look for gross rearrangement or partial inheritance. Dot hybridization and restriction analysis of 54 offspring of MaK-67 revealed the quantitative inheritance of an identical restriction pattern for two generations (Figure 4). We conclude that in this pedigree, DNA rearrangement is not a likely explanation for differential expression, with the proviso that rearrangement of a single expressing copy of the MK gene might go undetected against the large background of nonexpressing copies. In the MyK-84 pedigree we have detected loss of MK genes between generations, a result that might be anticipated because of the tandem array. Thus preferential loss of a few active copies of the MK gene could explain the decrease in activity in offspring MyK-84-8 and MyK-84-9 (Figure 7).

#### DNA Methylation

Another possible explanation for the variation in MK gene expression between generations, which we have begun to explore, is that DNA methylation is involved. There is a precedent for considering this possibility, because MT-1 gene expression appears to be con-

trolled in such a manner in some cells (Compere and Palmiter, 1981), and methylation changes in some immunoglobulin, globin and viral genes correlate with their expression (Shen and Maniatis, 1980; van der Ploeg et al., 1980; Groudine et al., 1981; Weintraub et al., 1981; Yagi and Koshland, 1981). We do not yet know which methylation sites are important for MT-1 gene expression, so we cannot direct our experiments towards likely control sites in pMK. Instead, we have assayed a variety of restriction sites within pMK for which there are methylation-sensitive enzymes.

We can summarize the data obtained thus far by noting that foreign DNA injected into mouse eggs can be methylated at many sites at some point during development; thus there must be a *de novo* methylation system that is independent of passage through the germ line or of meiosis. In contrast with these results, transfection of plasmids into L cells rarely results in *de novo* methylation, although the maintenance methylation system is quite effective (Pollack et al., 1980; Wigler et al., 1981; Stein et al., 1982). Chromosomal location appears to be an important feature of the *de novo* methylation system, since a particular sequence is not necessarily methylated in all animals. The methylation pattern acquired by the experimental animals is inherited in some cases (Figure 5) and lost in others (Figures 9 and 10). In the case of two offspring of MyK-84, MyK-84-3 and MyK-84-7, there is a correlation between a loss of essentially all methyl groups from sites that were partially methylated in the mother and an increase in HSV thymidine kinase activity (summarized in Figure 7).

The data presented here suggest that some fundamental event occurs during passage of a foreign gene from a transgenic mouse to its offspring that affects its ability to be expressed. We do not know when this change occurs, but a likely possibility is that it is associated with the generation of germ cells. We have observed extinction, repression and enhancement of MK gene activity in different offspring. It is too early to know whether gene expression will remain stable during subsequent generations. The most baffling aspect of this phenomenon is that the MK gene expression is so variable in comparisons of different offspring of the same mouse or of offspring of different mice. Chromosomal position effects may account for the latter variability, but the complete or partial loss of MK gene expression in offspring of MaK-67 is difficult to explain with our current understanding of gene commitment. We looked for changes in DNA arrangement or methylation, and found none in offspring of MaK-67 that were striking; however, in offspring of MyK-84 there was a rather striking difference in both of these parameters, with a tendency towards loss of MK genes to be associated with maintenance of high levels of thymidine kinase expression. There were too many copies of the MK gene in both of these animals for detailed analysis of this phenomenon. Neverthe-

less, the data indicate that achieving gene expression from microinjected genes does not ensure stable inheritance of that expression. It will be important to analyze gene expression in congenic mice with single copies of foreign genes, to try to discern the molecular basis of this phenomenon.

#### Experimental Procedures

##### Manipulation of Animals and Eggs

Fertilized eggs (C57 × SJL) were isolated and injected with 200–600 copies of plasmid and then reinserted into pseudopregnant surrogate mothers as described by Brinster et al. (1981). Shortly after weaning, the terminal quarter of the tail (50–100 mg) was surgically removed and homogenized with a Polytron (Brinkmann) in 5 ml of 1× SET (1% SDS, 5 mM EDTA, 10 mM Tris [pH 8]) containing 50 µg/ml proteinase K. Total nucleic acids were isolated and assayed for the presence of pMK as described below. Those mice that retained pMK sequences were analyzed for expression. Typically, 1 mg/kg body weight CdSO<sub>4</sub> was injected subcutaneously, and 18 hr later a partial hepatectomy (~150 mg) was performed with sodium pentobarbital anesthesia. Part of the tissue was used for thymidine kinase assay; the remainder was homogenized in 1× SET plus 200 µg/ml proteinase K. Up to three sequential partial hepatectomies have been performed at intervals ranging from 2–4 weeks. In some cases dexamethasone (0.5 mg/kg) was administered as equal amounts of a soluble and an insoluble preparation (Hager and Palmiter, 1981). More recently, we have given injections 18 and 4 hr before the operation, assuming that the MK mRNA is relatively unstable, while the thymidine kinase enzyme is relatively stable; thus this regimen should optimize the chances of detecting both mRNA and enzyme.

##### Isolation and Analysis of Nucleic Acids

Total nucleic acids were isolated by phenol–chloroform extraction and ethanol precipitation. DNA concentration was measured by the fluorescence assay described by Labarca and Paigen (1980) with a Perkin-Elmer Model 650-10M fluorescence spectrophotometer. For dot hybridization, total nucleic acids containing up to 20 µg DNA were precipitated with ethanol and dissolved by addition of 10 µl of 2 M NaCl, 0.1 M NaOH and boiling for 2 min. Aliquots (5 µl) were spotted directly onto dry nitrocellulose placed on Parafilm. A trace of ethanol in the sample facilitates even distribution of the DNA. The cellulose was air-dried, washed briefly in 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate [pH 7.0]) and then baked for 2 hr at 80°C. For restriction enzyme analysis, total nucleic acids were digested with RNAase A (100 µg/ml) for 30 min at 37°C, followed by digestion with proteinase K in the presence of SDS. The DNA was then extracted with phenol–chloroform and precipitated with ethanol. Aliquots (4–10 µg DNA) were digested in 40 µl of the buffer recommended by the manufacturer with about 2 U restriction enzyme per microgram of DNA for 6–18 hr. Samples were subjected to electrophoresis on agarose in a horizontal apparatus until the bromophenol blue marker had traversed about 13 cm. DNA was blotted onto nitrocellulose (Schleicher and Schuell) essentially as described by Southern (1975).

##### Plasmid Manipulation

Plasmid pMK was constructed as described by Brinster et al. (1981). The linear form was generated by digestion with Bam HI. In some experiments this linearized pMK was ligated to a sixfold weight excess of Bam HI-digested mouse DNA with T4 ligase. Analysis of the products by agarose gel electrophoresis revealed oligomers up to 30 kb long. The 2.1 kb Bst EII fragment was isolated by restriction of pMK with Bst EII followed by addition of DNA polymerase (Klenow fragment) and all four deoxynucleotide triphosphates. The products were subjected to electrophoresis on an agarose gel, and the 2.1 kb band was isolated by the NaClO<sub>4</sub>-glass fiber filter method of Chen and Thomas (1980).

##### Nick Translation and Hybridization Conditions

DNA fragments for nick translation were isolated from agarose gels by the method of Chen and Thomas (1980), and the DNA concentration was determined (Labarca and Paigen, 1980). DNA (15–20 ng) was nick-translated in a 10 µl reaction mixture containing 20 mM Tris (pH 7.5), 10 mM MgCl<sub>2</sub>, 0.3 mM CaCl<sub>2</sub>, 1 mM dithiothreitol, 100 ng DNA polymerase I (a gift from L. Loeb), 200 pg DNAase I, 40 µCi of one <sup>32</sup>P-labeled deoxyribonucleotide triphosphates (~2000 Ci/mmole; Amersham) and 0.15 mM of each of the other deoxyribonucleotide triphosphates. After 45 min of incubation at room temperature, EGTA was added to a concentration of 1 mM, NAD was added to a concentration of 50 µM, 2 U E. coli DNA ligase (New England BioLabs) was added and the reaction was continued for 15 min. Then 200 µl of 0.5× SET was added along with 10 µg herring sperm DNA, and the products were centrifuged through a 1.5 ml Sephadex G-75 column to isolate the high molecular weight products. The yield was typically 20 to 40 × 10<sup>6</sup> cpm, with a specific activity of 1 to 2 × 10<sup>6</sup> cpm/ng DNA. Hybridizations were performed at 45°C in Seal-a-meal bags at a concentration of 1 to 2 × 10<sup>6</sup> cpm of probe per milliliter in the presence of 50% formamide and 10% dextran sulfate essentially as described by Wahl et al. (1979). After hybridization the nitrocellulose was washed in 2× SSC plus 0.5× SET at 68°C for 1 hr with several changes, and then in 0.5× SET at 50°C with several changes of the wash solution. The nitrocellulose was then air-dried and allowed to expose X-Omat XAR-5 film at –70°C with a Lightning-Plus intensifying screen.

##### Other Procedures

Thymidine kinase activity was assayed as described by Brinster et al. (1981), and is expressed as counts per minute of TMP formed per minute per microgram wet weight of liver. MT-I mRNA was measured by solution hybridization as described by Beach and Palmiter (1981).

##### Acknowledgments

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## GENETIC ENGINEERING OF LABORATORY AND LIVESTOCK MAMMALS

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### Summary

Recent advances in recombinant genetics have made possible the transfer of cloned genes from one organism to the genome of another. Research with mice made transgenic by insertion of rat or human genes has provided direct evidence that transferred genes can be incorporated into the germline and expressed in the recipient. Current technology for gene transfer involves microinjection of the recombinant genes into the male pronucleus of the zygote. Resulting transgenic mice, when mated as adults, produced offspring that contained and expressed the transgenes. These observations serve as indications of the possibilities that exist for genetic engineering in livestock species. Although there are some technical problems to be overcome before livestock embryos can be genetically altered by these means, the genes for producing growth hormone transgenic livestock are currently available, and research groups are working toward this objective. In addition to this work with growth hormone genes, there are many other potential applications for genetic engineering livestock to produce more highly efficient production; however, there is considerable research to be done before the full potential of this technology can be achieved. It will be necessary to identify other genes that have potential for improving the production efficiency of livestock, and it will be necessary to gain a more complete understanding of the developmental and molecular biology of livestock. The potential impact of this technology in farm animal

production is enormous, but, in the short term, it will be a costly endeavor.

(Key Words: Genetics, Molecular Biology, Genetic Engineering, Growth, Mammals, Production.)

### Introduction

The history of agriculture is largely a history of genetic engineering. From the earliest efforts in agriculture, man has practiced genetic selection, often inadvertently, to produce choice agricultural products. Within the past 100 yr, geneticists have established protocols for scientific selection for desired traits, and a great deal of progress has been made in animal productivity. Nevertheless, animal scientists and livestock producers are interested in further improvements. The various disciplines within animal science are devoted to achieving these improvements, but progress seems slow compared with the desired result.

Within the past 3 yr, the potential for a dramatic rate of change has been created using a molecular approach to genetic engineering of mammals. Cloned genes have been incorporated into the genome of laboratory mice (Gordon et al., 1980; Brinster et al., 1981; Constantini and Lacy, 1981; Gordon and Ruddle, 1981; Wagner et al., 1981a,b; Palmiter et al., 1982; Wagner et al., 1982; Palmiter et al., 1983). These studies demonstrate that foreign genes can be expressed by the transgenic animals, and the technology is now available for use in livestock species. In this paper we review the progress in molecular genetic engineering of mammals and discuss possibilities for its use in livestock species.

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### Gene Recombination in Laboratory Animals

Recombinant genetic techniques allow the



transfer of specific cloned genes from the genome of one organism to the genetic makeup of another. Utilizing bacterial plasmids, gene recombination was first achieved in bacteria (Cohen et al., 1972) and later in simple eukaryotic species such as yeasts (Beggs, 1978). During the decade following the first successful gene transfers in bacteria, a major effort was mounted to achieve gene recombination in higher eukaryotic organisms including plants and animals. Until 1981, gene recombination in mammals was restricted to the transfer of genes into mammalian cells in tissue culture. The first successful transfer of a cloned gene into the genome of a mammalian cell line was accomplished by Paul Berg, using a recombinant SC40 virus genome containing an insert with the rabbit  $\beta$ -globin genomic clone ligated into the viral genome (Mulligan et al., 1979). Balb 3T3 mouse cells were transformed with the recombinant mammalian virus and transformed cells selected in media that allowed only the growth of the viral transformed cells. Only 1 in  $10^5$  cells were transformed and these cells were oncogenically transformed as well as genetically transformed. The resulting line of SV40 recombinant viral-transformed cells contained the rabbit  $\beta$ -globin gene and produced rabbit  $\beta$ -globin proteins (Mulligan et al., 1979). A major improvement in the methodology for the genetic transformation of mammalian cells in culture was developed by Wigler et al. (1979). Using the Herpes virus thymidine kinase (TK) gene, mouse L cells deficient in the TK gene function were transformed by exposure to a  $\text{CaPO}_4$  precipitate of the cloned Herpes TK gene. The 1 in  $10^7$  to  $10^9$  cells that take up and are genetically transformed by the Herpes gene are selected by growth in HAT media (media that interferes with nucleotide biosynthesis in deficient  $\text{TK}^-$  mouse L cell line). This TK selection method may be used to genetically transform  $\text{TK}^-$  cells with any cloned gene by co-transformation with the Herpes viral TK gene (Wigler et al., 1980). Because on entry into the nucleus of the recipient cell, the cloned DNA fragments are randomly ligated together to form a DNA polymer containing a mixture of the cloned DNA segments linked end-to-end, transformation with Herpes TK gene and any other gene results in the chromosomal integration of both genes linked together yielding TK selection and stable transformation with the desired gene.

It has long been the goal of molecular and

developmental biologists to introduce cloned genes into the germline of complete mammals in order to examine the interrelationships between the expression of genes and their regulation during development. Also, the optimum system for the study of the native function of a cloned and well characterized gene would clearly be the whole animal. Gene transfer in whole mammals would also allow the animal scientist to specifically alter the genetic makeup of livestock species with a selectivity unapproachable by traditional genetic selective procedures.

Germline gene recombination in complete animals must be approached by the genetic transformation of early embryonic cells from which a recombinant animal may develop. Because these cells may not be collected in large numbers and may not be propagated over long periods of time, the techniques used to genetically transform mammalian cells in culture cannot be used. In 1981, several laboratories successfully accomplished the germline genetic transformation of laboratory mice by microinjection of cloned DNA into the pronuclei of fertilized mouse eggs at the one-cell stage. The importance of using a single cell was to reduce chances of producing mosaic mice and ensure foreign gene integration into all cells of the developing animal. The relatively large pronuclear regions of the fertilized eggs also provided an easy target for microinjection. In addition to these reasons for pronuclear microinjection, Wagner et al. (1981a) suggested that the early male pronucleus may provide a highly specialized nuclear environment for the incorporation of DNA sequences and for their inclusion into a functional chromosomal region. The period of time during which sperm chromatin loses its protamine regulated structure and acquires a maternally determined histone structure may be a fortuitous time for incorporation of foreign DNA into a structure that is transcriptionally active. The molecular events within developing pronuclei were first investigated in the sea urchin. It has been suggested that sperm chromatin dispersion and male chromosomal gene expression may be manifestations of changes in the nucleoprotein content of the paternally derived chromatin within the developing male pronucleus. Kunkle et al. (1978) have shown that soon after fertilization of the sea urchin egg, the male pronuclear chromatin acquires proteins, probably maternally inherited, of molecu-

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lar weights greater than 80,000 daltons and a nuclear protein composition similar to that of the female pronucleus. These researchers postulate that such changes in male pronucleus composition may allow the paternal genome to participate in RNA synthesis (Longo and Kunkle, 1977). Recently Perreault et al. (1984) have detailed some of the molecular events in mammalian pronuclear development. Also, Laskey et al. (1977a,b, 1978) have isolated a group of enzymes from the frog oocyte that organize cellular histones into the nucleosomal chromatin units and that may function in structuring the sperm chromatin into transcriptionally functional chromosomal units during early male pronuclear development. Both of these observations suggest that extensive "rebuilding" of functional chromosomes occur within the early male pronucleus after removal of protamines from the sperm DNA during sperm decondensation. Therefore, similar early molecular events within the mouse male pronucleus might assist in assuring an appropriate nucleoprotein structure for the microinjected DNA and integration of exogenous DNA sequences placed into the early male pronucleus. Although this rationale for microinjection into the early male pronucleus may be supported by some molecular data in nonmammalian sys-

tems, no definitive proof that pronuclear or male pronuclear microinjection is advantageous has, as yet, been presented. Also, unpublished data suggests that gene transfer may, in some cases, be accomplished by late pronuclear or two-cell nuclear injections.

Zygote microinjection is carried out using two micromanipulators controlling two micropipettes. One-cell embryos, maintained in a microdrop of embryo culture media, are held individually at the polar body by suction applied via a microsyringe attached to a fire polished micropipette (25  $\mu$ m id, 80  $\mu$ m od). Injection is accomplished using an injection micropipette (.7 to 1.2  $\mu$ m od) attached to a .5-ml, screw driven, microsyringe to deliver 1 pl of DNA solution into the male pronucleus, oriented approximately 180° from the polar body. Successful injection is monitored by observation of an expansion of the pronuclear volume by approximately 25%. Microinjection is performed under 400x magnification using an inverted microscope. Embryos are held in a specially constructed depression slide above a sterile cover slip. The equipment for microinjection is shown in figure 1. A photomicrograph of the microinjection of a mouse egg is shown in figure 2.

Mice derived from one-cell embryos micro-

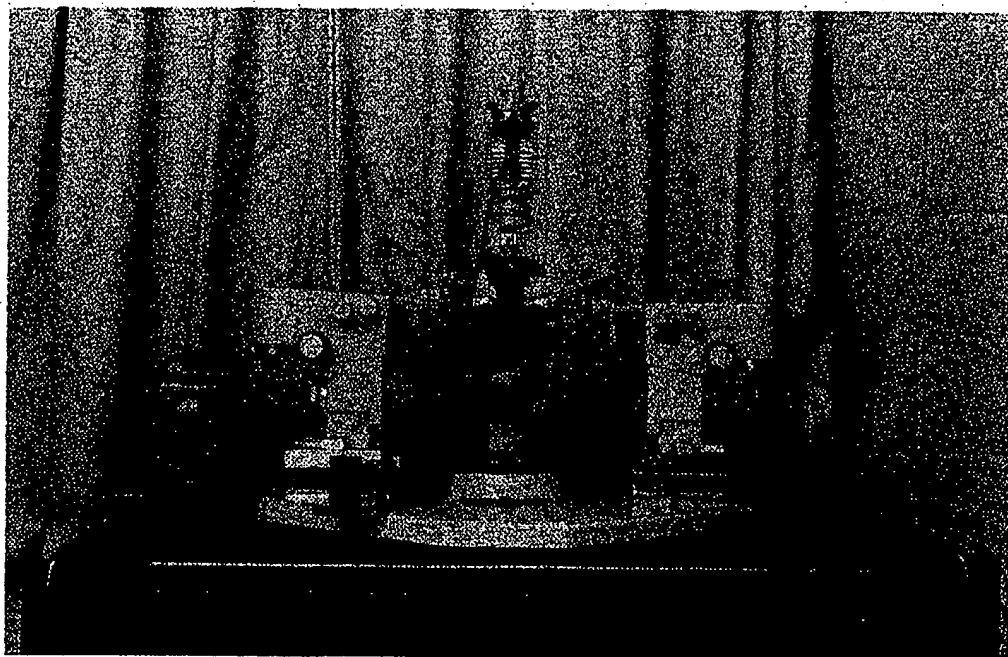


Figure 1. Leitz microinjection apparatus used in zygote-pronuclear microinjection procedures.

injected with specific cloned genes have been scrutinized on the molecular level. Experiments for determining the copy number, integrity, stability of integration and germline transmission of the introduced genes have been performed to investigate the fate of the foreign DNA. Some laboratories have also tried to determine the functional expression of the incorporated DNA.

The first successful incorporation of a cloned foreign gene into mice was reported by Gordon et al. (1980). In this study, a construct containing the SV40 virus origin of replication, pBR<sub>322</sub> plasmid sequences and the Herpes virus TK gene were introduced by microinjection. Southern blot hybridization showed fragments of this gene construction rearranged in the chromosomes of several mice. Almost simultaneously, several other laboratories announced the successful incorporation of other specific cloned genes into mice by DNA microinjection into fertilized embryos (Brinster et al., 1981; Constantini and Lacy, 1981; Gordon and Ruddle, 1981; Wagner et al., 1981a,b). From

these experiments there are now mouse lines that contain stably integrated SV40 DNA, Herpes Simplex Virus thymidine kinase (HSV-TK) gene, the human leukocyte interferon gene, the rabbit  $\beta$ -globin gene and the human  $\beta$ -globin gene. Gordon and Ruddle (1981) and Wagner et al. (1981b) demonstrated by Southern hybridization studies on undigested DNA from fetuses and live mice derived from injected embryos that the injected genes were stably integrated into mouse chromosomes. Constantini and Lacy (1981) also established chromosomal integration and determined the site of chromosomal integration for injected rabbit  $\beta$ -globin genes by in situ hybridization.

These results indicate that the cloned DNA has recombined in some manner with the host chromosomal DNA and is stably integrated. If this were the case, germline transmission of the DNA would be expected and hence, the establishment of lines of transgenic mice for studies of foreign gene function. Constantini and Lacy (1981) analyzed offspring from four male mice that contained rabbit  $\beta$ -globin genes and

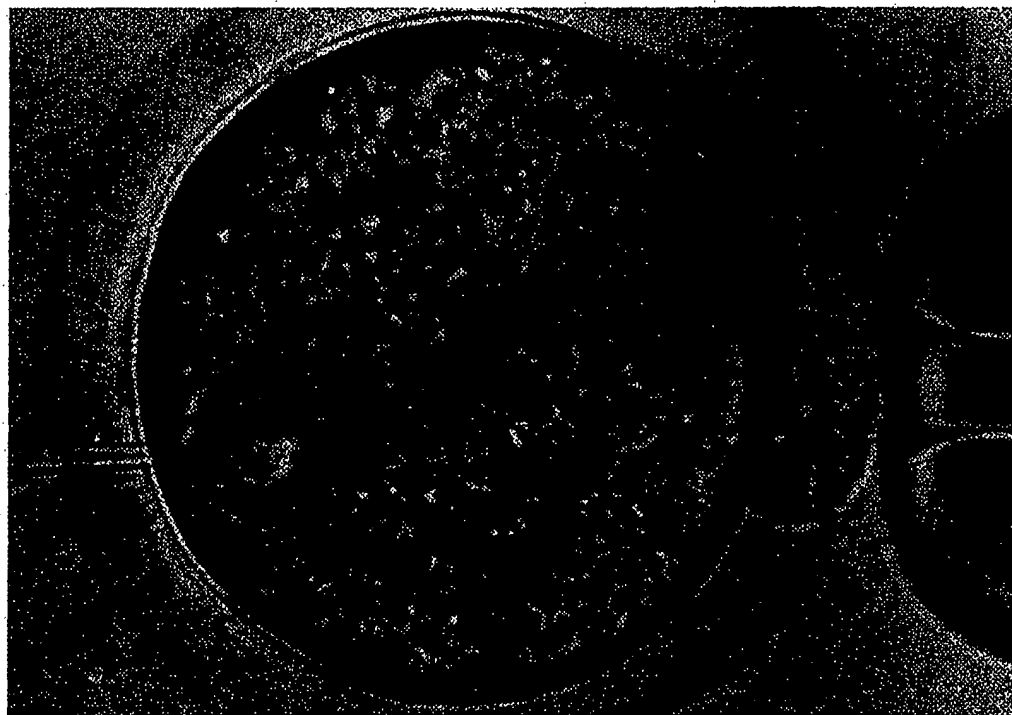


Figure 2. Photomicrograph showing the microinjection of a pronuclear mouse egg. The egg is held by a 60  $\mu$ m fire polished holding pipette and injected with a beveled 1.5  $\mu$ m injection pipette.

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showed by Southern hybridization that a portion of the group had complementary sequences within their genomic DNA. Wagner et al. (1981a) found the presence of rabbit  $\beta$ -globin protein in the blood of offspring of injected embryos. Transmission of human interferon genes in mice developed from embryos microinjected with this gene was also seen by Gordon and Ruddle (1981). Brinster et al. (1981), using a mouse metallothionein/HSV-TK fusion gene, also demonstrated the presence of the gene in second generation mice and the presence of the HSV-TK protein as well. These results suggest stable integration and Mendelian germline transmission of the foreign added gene in gene transfer in laboratory mice.

The main thrust of this line of research is to establish lines of animals that not only contain stably integrated foreign DNA, but ones that also express the inserted genes. Wagner et al. (1981) have demonstrated expression of injectable rabbit  $\beta$ -globin sequences in their mice.

The stable integration of intact foreign genes into the mouse genome allows analysis of foreign gene expression in these mouse lines. Wagner et al. (1981a) found that this was indeed the case for some of the rabbit  $\beta$ -globin genes present in transgenic mice containing rabbit  $\beta$ -globin genes. Immunodiffusion analysis showed the presence of a protein in the blood of these mice that crossreacted with mouse antirabbit globin antisera. This reaction was not seen in control animals and could be reversed by immunoabsorption of the antisera with rabbit globin. This finding was further corroborated by two-dimensional protein gel electrophoresis of the transgenic mouse hemoglobin demonstrating the presence of a globin species co-migrating with rabbit globin (Wagner et al., 1982). In addition, rabbit globin mRNA was detected in the bone marrow of these mice by Northern hybridization (Wagner et al., 1982).

Constantini and Lacy (1981), using a different construction of the same rabbit  $\beta$ -globin gene, did not observe the rabbit globin protein in their mice, but demonstrated the presence of rabbit  $\beta$ -globin mRNA in muscle tissue.

The difference in results between Constantini and Lacy (1981) and Wagner et al. (1981a) highlights the present lack of understanding regarding the control of gene expression in animal systems. Clearly, the function and expression of the  $\beta$ -globin gene depends upon more than the DNA sequences introduced into these lines of mice. Chromosomal position, flanking

DNA sequences or other factors may be of importance in gene function. It has been suggested that control of the expression of developmentally controlled genes may involve at least two determinants; the upstream flanking DNA sequences involved in promotion-regulation and the local DNA environment in the region of the chromosome where the gene resides (Jaenish et al., 1981). Until the role of nonadjoining DNA sequences can be properly evaluated, gene transfer with complex, developmentally controlled genes may be a "hit-or-miss" proposition.

Palmiter et al. (1982) have devised an elegant method to avoid the problems of gene expression in complex, developmentally controlled genes. These researchers have used the mouse metallothionein-1 gene promoter-regulatory sequences to act as a promoter of gene expression for almost any structural gene of interest in the mouse. Because the metallothionein-1 gene does not appear to be under the complex control found with other genes coding for proteins with more complex functions, this promoter appears to function well in any chromosomal location or condition. By fusing the metallothionein-1 gene promoter-regulator to the structural genes of interest, high levels of expression of the desired genes may be achieved. Initially, Brinster et al. (1981) used the metallothionein-1 (MT) promoter to express the HSV-TK gene in a MT-TK fusion gene injected into mouse embryos. The resulting mice produce substantial amounts of the TK protein. The universal value of the MT promoter was demonstrated by Palmiter et al. (1982) by fusing the MT promoter to the rat growth hormone (rGH) structural gene at the transcriptional initiation site and introducing their fusion gene into the germline of mice by zygote microinjection. Of the 21 mice that developed from these zygotes, seven carried the fusion gene and six of these grew significantly larger than their littermates. Several of these transgenic mice has extraordinarily high levels of the fusion mRNA in their liver and growth hormone (GH) in their serum. Not only does the approach of Palmiter et al. (1982) have implications for the study of the biology of GH in intact laboratory animals, but it may provide a way to deistically accelerate animal growth.

For GH transgenic animals to be economically useful for accelerated growth or for other functions, the transgenes must be stably integrated, transmitted and expressed in sub-



ield by a 60  $\mu$ m

sequent generations. Otherwise, the cost of producing the transgenic animals by current technology would be orders of magnitude greater than the gain that could possibly be realized. In this regard, Palmiter et al. (1983) demonstrated that GH transgenic mice do transmit the transgenes to their offspring in Mendelian fashion. When transgenic mice, expressing the transgenes and exhibiting accelerated growth, were outbred, approximately one-half of the offspring inherited the transgenes and grew at an accelerated rate (comparable to their transgenic parent) while the other one-half of the offspring did neither. This important contribution, as well as the report of Wagner et al. (1981a) involving offspring of  $\beta$ -globin transgenic mice, confirm the feasibility of producing lines of transgenic animals with improved production efficiencies.

In order to apply the procedure of gene transfer to livestock species, it is necessary to establish efficient procedures for the recovery of pronuclear livestock embryos, observation of their pronuclei for effective microinjection and to transfer these injected eggs into recipient females with an overall efficiency approaching that now available in laboratory mice. The cloning of livestock genes is well underway and those genes of greatest interest are available or will be available in the near future. The bovine GH (bGH) gene has recently been cloned by Woychik et al. (1982) and efforts to introduce this and other GH genes are underway in our laboratories as well as the laboratories of others.

**Problems in Animal Sciences That May Be Addressable Through Recombinant Genetics**

*Growth Efficiency.* Traditionally, the great-

est research emphasis in the animal sciences has been, and continues to be, growth efficiency, with particular priority on feed efficiency, rate of gain and body composition. The reason for this is obvious because feed is the major single variable cost of production. Growth efficiency in farm animals has increased substantially over the past 50 yr (Smith et al., 1980) by both improved nutrition and superior genetics of breeding stock. While it is clear that many important traits relating to growth efficiency are heritable, continued improvements by traditional animal breeding methods will depend on the range of genetic variability in current breeds. Nutritional improvements have taken advantage of the improved genetic capabilities of the species and now diets more adequately meet the nutrient requirements of animals at various physiological phases of growth and reproduction. While it is clear that both disciplines will continue to improve growth efficiency, only incremental increases are foreseeable with these conventional approaches.

The question of the likelihood of achieving a dramatic improvement in growth and feed efficiency through recombinant genetics is relevant. Studies dating to those of Evans and Simpson (1931) have demonstrated that long term treatment of animals with growth hormone (GH) or adenohipophysis extracts can substantially improve both growth rate and feed efficiency in animals. Evans and Simpson (1931) found that at 200 d of age, female rats receiving chronic daily injections of an extract from the anterior pituitary gland, rich in GH, reached approximately 475 g while controls were approximately 270 g in weight (table 1). At the same age, male rats weighed 530 and 410 g, respectively. At 400 d of age, GH-treated females weighed about 570 g while controls

**TABLE 1. EFFECT OF BOVINE GROWTH HORMONE PREPARATION ON GROWTH IN RATS**

Sex	Treat- ment	Days of age				
		30	100	200	300	400
		Weight <sup>a</sup> , g				
Female	Control	70	230	270	270	240
Female	GH	70	350	475	530	570
Male	Control	60	300	410	500	520
Male	GH	60	360	530	750	850

<sup>a</sup>Approximate values derived from figures presented by Evans and Simpson (1931).

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<sup>a</sup>Values 224 d of age

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\*P<.05

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weighed 240 g. At the same age, males weighed 850 and 520 g, respectively. These are dramatic increases in growth. Evans and Simpson also noted that the effect of GH on body weight in rats was not particularly evident until after the animals had reached 100 d of age. Thus, this early study provides evidence that the physiological stage on the growth curve of animals is important in determining the degree of response to exogenous GH. Therefore, use of GH in young animals might be expected to yield poor response.

Lee and Schaffer (1934) performed experiments similar to those reported by Evans and Simpson (1931), but they also evaluated treatment effects on body composition of experimental rats (table 2). They found, in pair feeding experiments, that while consuming the same quantity of feed as controls, rats treated with pituitary gland extracts gained more weight than did the controls. At the same time, body composition revealed an increase in water, nitrogen and ash content, but a decrease in fat (ether extract) content. Even with small numbers of animals, it is evident from the data presented that GH exerted little effect on the younger rats (rats started on treatment at 52 d of age and slaughtered at 129 d of age gained a mean net of -2.5 g, while rats started at 171 d of age and slaughtered at 227 d of age gained a mean net of 45 g in response to GH). This is

potentially an important point, for it suggests that the response to increased GH may depend on age at treatment. In consideration of the results of both Evans and Simpson (1931) and Lee and Schaffer (1934), it has been clear for half a century that daily treatment with exogenous GH can greatly increase nonfat body growth in experimental animals.

Presumably due to lack of available hormone in sufficient quantity, studies of the effect of exogenous GH on farm species were done only recently. Machlin (1972) reported that exogenous porcine GH (pGH) positively affected growth in pigs; however, high doses equivalent to those used in the rat studies proved toxic to pigs. In general, the results with lower doses of pGH revealed increased muscle growth and decreased fat deposition (table 3). Five experiments with various numbers of pigs were reported, and effects of pGH on rate of gain and feed efficiency were significantly improved in some experiments. This study makes evident two especially important points: 1) pigs are adversely sensitive to very high levels of exogenous GH, and 2) muscle growth, in particular, in pigs can be increased by the hormone. Therefore, moderately increased levels of GH in the pig would appear to be advantageous if the cost of hormone and its administration did not override the benefits to be gained. Until large quantities of inexpensive, pure pGH are available

TABLE 2. EFFECT OF BOVINE GROWTH HORMONE (GH) PREPARATION ON WEIGHT AND BODY COMPOSITION OF PAIR-FED RATS

Measurement <sup>a</sup>	Control	GH-treated	Difference due to GH <sup>b</sup> , g
No.	12	12	
Initial wt, g	198	196	
Final wt, g	262	304	42**
Empty carcass wt, g	250	290	40**
Empty carcass composition, % of carcass wt			
Water	58	62	36**
Fat	19	13	-11*
Nitrogen	3.0	3.2	1.8**
Ash	4.0	4.1	1.7**

<sup>a</sup>Values derived from table 3, Lee and Schaffer (1934). Pairs varied in age at initiation of treatment (52 to 224 d of age) and age at end of slaughter (129 to 287 d of age).

<sup>b</sup>Values derived from table 6, Lee and Schaffer (1934).

\*P<.05.

\*\*P<.01.

TABLE 3. EFFECT OF EXOGENOUS PORCINE GROWTH HORMONE (pGH) ON GROWTH AND CARCASS CHARACTERISTICS OF SWINE<sup>a</sup>

Item	Exogenous pGH (mg·kg body wt <sup>-1</sup> ·d <sup>-1</sup> )	
	0	.13
No. of animals	18	18
Initial wt, kg	45.9	46.0
Slaughter wt, kg	94.3	94.0
Avg daily gain, kg	.74	.86**
Feed/gain	3.33	2.89*
Carcass		
Dressing percentage	71.5	68.9**
Backfat, cm	3.48	2.79***
Loin eye, cm <sup>2</sup>	25.8	31.9***

<sup>a</sup>Experiment 2 from Machlin (1972).

\*P&lt;.05.

\*\*P&lt;.01.

\*\*\*P&lt;.001.

with appropriate delivery systems, treatment of growing swine with exogenous GH will have little practical value.

Growth hormone treatment studies have also been performed in ruminants. Studies by Wheatley et al. (1966) and Reklewska (1974), the former with adult sheep, found no increase in growth of sheep in response to exogenous bovine GH (bGH). Moseley et al. (1982) reported that GH treatment of steers increased nitrogen retention, supporting the data from rats and pigs on increased muscle growth in response to exogenous GH. A recent report by Muir et al. (1983) also found no increase in growth of lambs in response to exogenous ovine GH; however, in this study, feed efficiency was significantly increased (by 7.4%). Wagner and Veenhuizen (1978) reported increases in both growth rate (20% increase) and feed efficiency (13.6% improvement) in lambs started at relatively large size (40 kg). With heifer calves, Brumby (1959) found that exogenous GH increased growth rate, but he did not report feed efficiency data. Thus, in ruminants, as in swine, increased levels of GH could prove advantageous if the costs were not prohibitive.

Currently, the advantages of exogenous GH treatment of livestock can not be realized because of the lack of sufficient, economical GH. However, this situation may change in the near future because a number of companies are plan-

ning to produce GH by large scale culture of recombinant microbes. Practical use of exogenous GH is likely to be labor intensive because daily treatment or sustained release implants will probably be required to achieve the desired effect. As an alternative, if the genetic capacity of livestock can be manipulated to produce extra endogenous GH with adequate specific regulation of expression so that particular production traits desired in a specific group of animals would be improved (in this case, growth of meat producing animals—but not necessarily their progenitors), a great advantage would be available to the livestock industry.

**Milk Production Efficiency.** Currently, and in the foreseeable future, there is an excess of milk produced in the United States and dairy producers are faced with minimal profits. While increased milk production per se might not be desirable now, a dramatic improvement in the efficiency (cost) of milk production would be of great benefit to producers and consumers in the face of continued excess milk supply. There is reason for optimism that recombinant DNA (rDNA) technology can be used to dramatically improve the efficiency of milk production.

A number of recent studies have demonstrated that exogenous GH treatment improved milk production efficiency (Brumby and Hancock, 1955; Bullis et al., 1965; Machlin, 1973; Bines et al., 1980; Peel et al., 1981,

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1982; Gorewit et al., 1982). With low producing dairy cattle, these studies demonstrated up to a 50% improvement in milk production with more milk produced per unit of feed consumed. Peel et al. (1981, 1982) used high producing dairy cows and observed significant (9.5 and 15.2%) increases in milk yield, with slight (nonsignificant) increases in feed efficiency in response to 51.5 IU/d exogenous bGH for short periods (10 to 11 d) early in lactation (10 to 18 wk). In these studies, yield of fat, lactose and protein were increased. Gorewit et al. (1982) using high producing cows in late lactation (~35 wk), found dramatic improvements in milk yield during two limited treatment periods with bGH. Milk yield in this study was improved by 31 to 35%, milk fat yield increased by 32 to 38%, milk protein yield increased by 18 to 22% and milk lactose yield increased by 34 to 37%. The studies of Peel et al. (1981, 1982) and Gorewit et al. (1982) suggest that stage of lactation and, thus, milk production rate of cows at the time of treatment greatly influences the response to exogenous GH.

In view of the potential for use of rDNA technology to insert exogenous genes into the genome of animals, it is within current capability to use this in dairy cattle. The limitation, in addition to the fundamental skill required to microinject pronuclei of bovine eggs, is to make a gene construct with a promoter controlled so that GH production would be regulated with respect to mammary gland function of the cow. Without such precise control, the inserted GH gene, if continually expressed to produce excess blood GH levels throughout adulthood, might cause gigantism, without or in addition to, increased milk production. Such a result would probably be undesirable. This circumstance requires that precise control of GH gene be incorporated into the construct and that control be experienced in the producing animal.

**Animal Health.** Currently, biotechnological applications in animal health are focused on development of vaccines with almost immediate target dates for utilization. These developments hold great potential for improved animal health; however, it is conceivable that rDNA technology can have even greater impact by the development of genetic constructs that would produce inherent specific disease resistance. It is intuitively obvious that most diseases are, at least to some degree, genetically predisposed because most diseases affect only a few species. Furthermore, resistance to some diseases is

known to be related to particular genetic loci, e.g., Marek's disease in poultry (Payne, 1973) and many human and mouse diseases (Bach, 1982). Another example of genetically determined disease is internal parasite resistance (e.g., Preston and Allonby, 1978). These diseases should be responsive to recombinant genetic approaches, involving insertion of the appropriate genes. The ability to perform these alterations will require considerable effort in domestic animal molecular biology to identify gene products and development of gene constructs, particularly those involved in non-specific disease control.

**Reproductive Efficiency.** It is generally recognized that the single greatest limitation to maximizing overall efficiency of animal production is reproductive efficiency (Cartwright et al., 1980). In some of the farm species, the current level of reproductive efficiency is less than 50% of the level that is possible. Little improvement in reproductive efficiency has been made through conventional animal breeding methods because heritability of reproductive traits is low (Smith et al., 1983).

Generally, advances in reproductive efficiency will depend on the identification of rate-limiting gene products. For example, gene products identified by Murray et al. (1978) and Segerson (1981) have the potential to improve embryonic survival in pigs, cattle and sheep and may find utility through rDNA approaches. These gene products are powerful immunosuppressive polypeptides produced by the uterus during the peri-implantation period. They would be natural agents promoting embryo survival through the implantation phase, which is the time during which the largest proportion of embryo loss occurs (Ulberg and Rampacek, 1974). As with other potential improvements through rDNA approaches, the benefits can be realized with no increased labor input and would be genetically transferred from generation-to-generation, once success is achieved.

**Use of Recombinant Animals for Physiological Study.** It is conceivable that genetically engineered animals might be useful as models in studies of specific gene product function in a manner analogous to hormone treatment experiments performed currently. The advantages to be gained with recombinant animals are that the inconvenience, stress, and imprecision associated with traditional animal experimentation might be overcome to a significant

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degree. In studies with recombinant animal models, laboratory animals might be made transgenic for a particular gene and, with regulated expression, the specific effects of the gene product could be evaluated separately from, or in association with, other related gene products. In their elegant studies on expression of metallothionein/rat and human GH fusion genes in mice, Palmiter et al. (1982, 1983) have presented information on a variety of aspects of GH function and regulation. These studies illustrate the concept that transgenic animals can be used effectively in physiological research. Although the list of currently available gene products from livestock species is short (essentially limited to peptide hormones, milk proteins, some secretory products, some enzymes and histocompatibility antigens), the potential list is huge. Each gene product would offer possibilities for study of physiological effects through production of specific transgenic animals. With the accumulation of this knowledge, the capacity to control and exploit multigene traits may be attained.

**The Promise of—  
and Problems Restricting Progress Towards—  
Recombinant Livestock**

Laboratory experiments performed during the last 3 yr clearly demonstrate that specific cloned genes may be transferred into the germline of mammals, and that these genes will function to produce their specific gene products, phenotypically altering the resulting recombinant animal. The animal used for these studies has been the laboratory mouse. Primarily, this is because a great deal is known about the genetic makeup of this animal and embryological procedures of handling and culturing mouse embryos are well established. Although all published experiments, to date, have used the mouse system, there are not substantial restrictions to utilizing other laboratory or livestock species in such experiments.

Although procedures for collection, handling and culture of livestock embryos are not as well established as those for the mouse, livestock embryology has advanced to the level where one-cell pronuclear eggs may be recovered, cultured, micromanipulated and transferred to recipient females with sufficient efficiency to allow gene transfer experiments in these species. Two major obstacles to successful gene transfer in livestock do remain: (1) the low effi-

ciency of the present gene transfer process, and (2) the dense, pigmented cytoplasm in most livestock embryos that makes the pronuclei, making injection difficult, if not impossible (figure 3). It seems likely that these obstacles will be overcome in the near future, opening the "door" to a revolution in livestock genetics.

Little is known of the developmental biology of domestic animals. This is important because the control of gene function varies with the developmental stage of the individual and with physiological status. Controlled expression of genes in laboratory animal development is reasonably well understood, but to date practically no research has been done in this critical area in livestock species. Thus the ability to control gene product production in relation to a particular physiological state, function, or organ system is critical if the desired effect is to be realized.

Another handicap to gene engineering in livestock species is the lack of knowledge of gene products important for functions related to production. It will be necessary to identify, characterize and isolate gene products important to animal production before the genes specifying these gene products can be isolated. This will necessitate reorientation of animal scientists or molecular biologists to carry out this important function. Without such knowledge, only a limited number of genes will be usefully available for gene transfer.

With regard to the limited set of genes available for use in livestock genetic engineering, an often-raised assertion is that, because most important production traits are controlled by multiple genes, genetic engineering will have little impact on most aspects of production. The response to this assertion must be offered at two levels. First, the multigene traits offer not only a greater challenge because the complexity of control of the trait is almost certainly greater than would be true to single gene traits, but the multigene traits probably offer greater opportunity for benefit because at least some of the genes could have additive and/or cooperative effects, if the entire trait is not controlled by a single gene (a "bottleneck" in a pathway). On the other hand, if the "bottleneck" situation exists, then once the limiting gene is identified the multigene trait would behave as a single gene trait until the next limiting gene function became restrictive. Clearly, this would be more complex and challenging than truly single gene functions, but there

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are benefits to be gained not only in terms of improved animal productivity, but also in the fundamental knowledge of the biology of livestock. The second response to the assertion is that the very first anticipated use of genetic engineering in livestock will be in an area—growth efficiency—that is certainly a multigenic trait. Several of the genes that are involved in growth are known, and it seems certain that more genes that influence growth efficiency ultimately will be identified. Some of the gene products involved in regulating growth efficiency are: GH, GH releasing factor, somatostatin, insulin-like growth factors, enzymes controlling steroid production, enzymes controlling thyroxine production, et cetera. Yet, as reviewed above, GH transgenic animals can be expected to have dramatically improved growth and milk production efficiency. As more knowledge in livestock genetics at the level of the gene, as opposed to the "trait" is obtained, the utility of genetic engineering of the various production functions will be more certain.

Promoters are the control portions of genes that allow structural genes to be expressed at the appropriate time and at an appropriate rate for the function of an organ in a particular physiological status. Thus, promoters not only initiate gene expression, but they also regulate the amount of expression that takes place

under a specific condition. Therefore, knowledge of a variety of gene promoters for use with gene transfer in livestock species will be a necessity. Eventually, it may be possible to program animals to perform differently in response to specific triggering mechanisms (such as feed additives, environmental factors, or semiochemicals) if promoters sensitive to the triggering mechanisms were inserted upstream from the structural transgenes. For example, an animal might be designed with GH genes, genes for increasing litter size and genes for resistance to internal parasites. If a sufficient variety of promoters were available and if promoter/structural gene constructs were specifically chosen, it might be possible to cause the female to grow to normal size at the normal rate, yet her offspring would have accelerated growth rates in response to a specific feed additive. At the time of breeding, a pheromone might be used to cause the promoter for uterine gene products responsible for enhancing embryo survival to be expressed. At periodic intervals, triggering agents (for example, photoperiod) might be used to promote parasite resistance. Therefore, with appropriate promoters, a variety of functions might be programmed into the same animal and expressed at the will of the livestock manager. However, it must be emphasized that these possibilities are relatively far



Figure 3. Sheep zygote. Cytoplasm of livestock animal zygotes is dense and pigmented, making observation of pronuclei difficult.

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While genetic engineering of animals presents opportunities, the cost and complexity are significant considerations. At present the genetic engineering programs underway involve personnel from several institutions bringing together the required expertise in several fields. Although the assembly of requisite expertise at one location would be a distinct advantage, cost and lack of availability of expert personnel has been a detriment. Several types of expertise focusing on production of transgenic animals is needed. A minimum combination of expertise would include mammalian rDNA technology (for isolation of structural genes and promoters); microbial rDNA technology (for cloning and multiplying genes); nucleic acid hybridization and blot transfer techniques; protein analytical techniques; developmental and physiological biology, retrovirology; embryo collection, culture, and manipulation; animal husbandry and animal performance analytical techniques. Some of these capabilities may be resident in a single person, but efficiency would require more than one person collaborating on some areas. Thus, it could be anticipated that a minimum of four or five scientists concentrating on the group objective would be a necessity. Cost of setting up and operating is substantial also. To start a program from "scratch" currently would require several hundred thousand dollars to equip and several thousand dollars per month to supply each investigator, assuming that some of the equipment would be shared. In addition, technical assistants and graduate students would add to the cost. Another consideration is that gene constructs and some aspects of the technology will be protected by patents.

Clearly, gene transfer and recombinant livestock offer a means to alter the fundamental genetic makeup of livestock to a greater extent, in a few decades, than may have been achieved in the entire past history of the science of livestock genetics. Those of us involved in this research look forward to the challenge and promise of this exciting new technology.

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# High Level, Regulated Expression of the Chimeric P-Enolpyruvate Carboxykinase (GTP)-Bacterial *O*<sup>6</sup>-Alkylguanine-DNA Alkyltransferase (*ada*) Gene in Transgenic Mice<sup>1</sup>

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## ABSTRACT

Transgenic animals expressing genes capable of repairing DNA may be a valuable tool to study the effect of DNA-damaging agents on tissue-specific carcinogenesis. For this reason, we constructed a chimeric gene consisting of the promoter-regulatory region of the phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) (PEPCK) gene linked to the *Escherichia coli ada* gene coding for *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase and the polyadenylate region from the bovine growth hormone gene. The PEPCK promoter results in gene expression in liver and kidney and is induced by hormones, and its transcription is regulated by diet. The chimeric PEPCK *ada* gene was injected into the male pronucleus of fertilized eggs to produce transgenic mice. Six of 65 developing mice contained 5-10 copies of the intact *trans* gene per genome. Two founders transmitted the *trans* gene in a heterozygous manner, whereas 3 transmitted as germ line mosaics and 1 did not transmit to F<sub>1</sub> offspring. All F<sub>1</sub> offspring carrying the PEPCK *ada trans* gene expressed *ada* mRNA in liver and kidney and produced a functional alkyltransferase with a protein molecular weight of 39,000 originating from the bacterial gene. Total alkyltransferase activity was increased in the liver of F<sub>1</sub> offspring from all founder mice, but offspring of only one founder had elevated renal alkyltransferase levels. A diet high in protein markedly increased *ada* mRNA and alkyltransferase activity within 1 week in both liver and kidney, whereas a high carbohydrate diet for 1 week markedly reduced expression of PEPCK *ada* and alkyltransferase levels. Nontransgenic animals were unaffected by these dietary manipulations. During induction with a high protein diet, hepatic alkyltransferase in transgenic mice was 16.6 ± 1.5 units/μg DNA (mean ± SE) compared to 5.3 ± 0.6 units/μg DNA in control animals. This level of alkyltransferase is higher than that in any mammalian tissue noted previously except human liver. Transgenic animals expressing high levels of alkyltransferase should help define the role of DNA repair in protection from carcinogenesis induced by *N*-nitroso compounds.

## INTRODUCTION

The tissue specificity of chemical carcinogenesis appears to depend on the overall level of DNA damage relative to the capacity for DNA repair. Perhaps the best studied example of this is the carcinogenicity of the *N*-nitroso compounds which damage DNA and induce tumors in specific rat and mouse tissues (1-3). One of the critical proteins responsible for repair of DNA damage induced by *N*-nitroso compounds is *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase (alkyltransferase) which repairs *O*<sup>6</sup>-alkylguanine lesions in double-stranded DNA (4, 5). Thus, compounds which directly damage DNA, such as the nitrosou-

reas, induce tumors in tissues which have a low capacity for repair of *O*<sup>6</sup>-alkylguanine-DNA adducts (e.g., brain, mammary tissue, bone marrow and thymus (6-9)). Other *N*-nitroso compounds, such as the nitrosamines, require metabolic activation to produce the proximate carcinogen (10). With these agents, tissues which enzymatically produce the proximate carcinogen, such as liver, kidney, and lung, are the targets for malignant transformation (11, 12). In many instances, the target tissues for nitrosamine carcinogenesis have higher alkyltransferase activity than brain, mammary tissue, or bone marrow but are able to activate the nitrosamine and thus develop high levels of DNA damage (11-13). In these tissues, carcinogenic doses are those that produce sufficient *O*<sup>6</sup>-alkylguanine lesions to overwhelm the alkyltransferase (1, 3, 11-13). While it appears that the alkyltransferase has an important role in protection from *N*-nitroso compound induced carcinogenesis, it is also clear that the relationship between DNA damage and tissue specific DNA repair in chemical carcinogenesis is complex. For this reason, it is important to analyze the carcinogenic process within defined systems where manipulation of a single component of the process can be studied.

Previous studies have documented that alterations in alkyltransferase activity can change cellular susceptibility to *N*-nitroso compounds *in vitro*. Decreasing the level of alkyltransferase in tissue culture cells using the specific alkyltransferase inhibitor, *O*<sup>6</sup>-methylguanine, increases the cytotoxicity, mutagenicity, and chromosomal aberrations caused by both methylating and chloroethylating nitrosoureas (14-16). To significantly increase alkyltransferase activity in mammalian cells, we and others have used gene transfer of the *Escherichia coli* alkyltransferase gene, *ada* (17-19). The bacterial alkyltransferase repairs *O*<sup>6</sup>-methylguanine adducts by a similar mechanism to that of the mammalian protein (20). Following *ada* gene transfer into mammalian cells, expression of the bacterial alkyltransferase results in resistance to the cytotoxicity and chromosomal aberrations of nitrosoureas (17-19). Cumulatively, the *in vivo* and *in vitro* data suggest that the alkyltransferase protects cells from the DNA-damaging effects of *N*-nitroso compounds. However it remains unknown whether increased expression of alkyltransferase could alter the carcinogenicity of *N*-nitroso compounds and decrease tumor induction in a tissue-specific manner.

Transgenic animals expressing DNA repair genes may be a valuable tool to define the effect of DNA-damaging agents on tissue-specific carcinogenesis. For this reason, we produced transgenic animals expressing the *ada* gene. To provide tissue specificity of *ada* gene expression, we utilized the inducible and tissue-specific mammalian promoter, PEPCK<sup>3</sup> (EC 4.1.1.32) (21, 22) to potentially target gene expression to the liver and

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<sup>3</sup> The abbreviations used are: PEPCK, phosphoenolpyruvate carboxykinase (GTP); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; poly(A), polyadenylate.

kidney. The PEPCK promoter is a well-characterized and unique promoter system which has defined glucocorticoid and cAMP response elements (23). Transcription from the PEPCK promoter is increased in the presence of glucocorticoids and cAMP and decreased by insulin in tissue culture cells (24, 25). In transgenic animals, the PEPCK promoter acts in the manner predicted by expression of endogenous PEPCK: when linked to the bovine growth hormone gene, the chimeric *trans* gene is efficiently expressed in a tissue-specific manner in liver and kidney and is regulated by diet and hormones (22). By utilizing the chimeric PEPCK *ada* gene, our aim was to target *ada* expression to liver and kidney and determine whether alkyltransferase activity in these tissues can be induced significantly above basal levels. Characterization of the expression and inducibility of the chimeric gene will enable us to then test the hypothesis that increased alkyltransferase activity will decrease carcinogenesis of *N*-nitroso compounds *in vivo*.

## MATERIALS AND METHODS

**Chemical Reagents.** Restriction endonucleases and other DNA-modifying enzymes were purchased from Boehringer Mannheim. The oligonucleotide-labeling kit was obtained from Pharmacia. [ $\alpha$ - $^{32}$ P]dCTP (3000 Ci/mmol) and Gene Screen Plus were obtained from New England Nuclear. Genetecin (G-418) was purchased from GIBCO. Guanidinium thiocyanate was obtained from Fluka and cesium chloride from Boehringer Mannheim. All of the other reagents used in the study were purchased from Sigma Chemical Co. or Pharmacia and were of the highest purity available.

**Animal Maintenance and Breeding.** Animals were fed a regular diet of Purina rodent chow and acidified water *ad libitum* and given standard fluorescent light illumination for a 12-h cycle each day. Certain animals were switched to either the high protein or high carbohydrate diet formula for either 1 or 4 weeks. Animals were killed by cervical dislocation while under ether anesthesia. The high protein diet contained 64% casein, 22%  $\alpha$ -cell nutrient fiber, 11% vegetable oil, 2% brewers' yeast, and a 1% mineral mix with vitamins. The high carbohydrate diet contained 81.5% sucrose, 12.2% casein, 0.3% DL-methionine, 4% cotton seed oil, 2% brewers' yeast, and a 1% mineral mix plus vitamins (Nutritional Biochemical Corp.).

Plasmids. pSV2*ada*alkB was kindly supplied by L. Samson, Harvard School of Public Health (19), and pBSM13 into which the 3' end of the bovine growth hormone gene had been inserted was the gift of F. Rottman, Case Western Reserve University School of Medicine. pPCKBH1.2 has been described (24). The 1.3-kilobase rat GAPDH complementary DNA fragment was isolated from pRGAPDH-1 (26).

**Plasmid Construction and Preparation of Recombinant DNA for Injection.** The *E. coli* alkyltransferase gene, *ada*, was isolated as the 1320-base pair *Hind*III-*Sma*I fragment from pSV2*ada*alkB. The pSV2*ada*alkB plasmid contains the *ada*-*alkB* operon which was originally isolated by Lemotte and Walker (27) from *E. coli* K-12. The 621-base pair *Bam*HI-*Bgl*II fragment isolated from pPCK BH1.2 contains the region from -548 to +73 of the rat PEPCK promoter-regulatory region. The PEPCK promoter and *ada* gene fragments were ligated together in the ClaI2N plasmid (28) and isolated following *Hind*III-*Sma*I digestion (Fig. 1). The 710-base pair *Sma*I-*Eco*RI fragment from the 3' end of the bovine growth hormone gene which includes a portion of the fifth exon and the poly(A) region (29) was ligated into pBS M13, and the PEPCK *ada* chimeric gene was then inserted 5' to the bovine growth hormone fragment at the *Hind*III-*Sma*I sites. The 2340-base pair *Pst*I-*Sph*I fragment which contains the PEPCK *ada* gene and 364 base pairs from the 710-base pair region of the bovine growth hormone gene [which includes the poly(A) sequences] was isolated by agarose gel electrophoresis, purified by phenol-chloroform extraction, passed through an Elutip filter (Schleicher and Schuell), and suspended in sterile water for injection at 15 ng/ $\mu$ l.

**Microinjection of Recombinant DNA into Single-Cell Embryos.** The

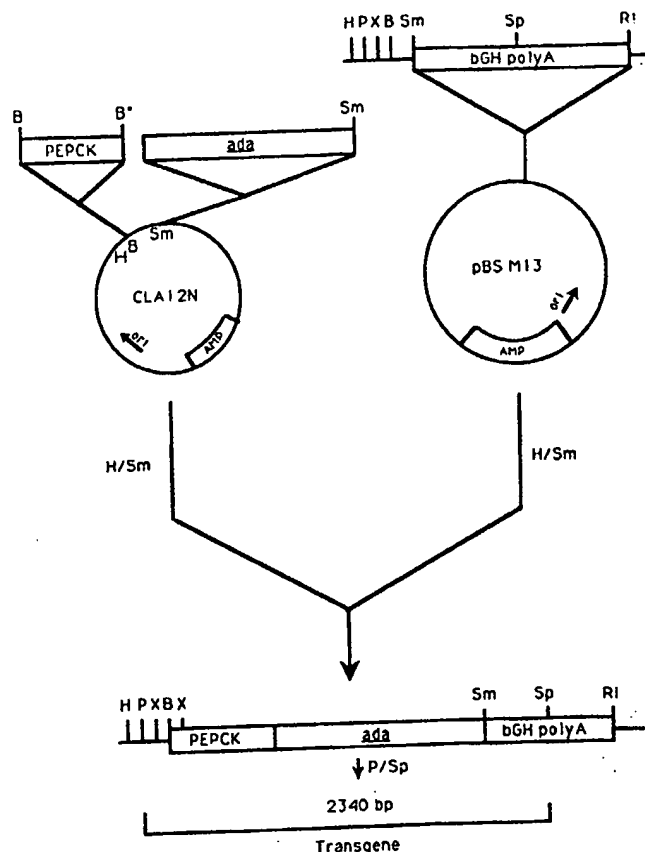


Fig. 1. Construction of the chimeric PEPCK *ada* gene. The 621-base pair *Bam*HI-*Bgl*II PEPCK promoter-regulatory region from pPCK BH1.2 was ligated to the 1320-base pair *Hind*III-*Sma*I *ada* gene from pSV2*ada*alkB in the ClaI2N plasmid. The 710-base pair *Sma*I-*Eco*RI fragment containing the 3' end of the bovine growth hormone gene including the poly(A) region (29) was inserted into the pBS M13 plasmid. The PEPCK *ada* gene was isolated following *Hind*III-*Sma*I digestion and ligated 5' to the bovine growth hormone fragment in pBS M13. The 2340-base pair *Pst*I-*Sph*I fragment containing PEPCK *ada* and 364 base pairs of the bovine growth hormone gene poly(A) signal was isolated and used for microinjection to produce the transgenic animals. B, *Bam*HI; B\*, *Bgl*II; H, *Hind*III; P, *Pst*I; RI, *Eco*RI; Sp, *Sph*I; Sm, *Sma*I; X, *Xba*I; bGH, bovine growth hormone; bp, base pairs.

procedure for microinjection of recombinant DNA has been described in detail (30). Briefly, the fertilized embryo was flushed from C57B/6XSJL F<sub>1</sub> mice 8 h after ovulation, and the pronucleus was injected with 2  $\mu$ l of DNA solution containing PEPCK *ada* after which the embryo was reimplanted in pseudopregnant mice (30). Offspring were weaned at 4 weeks and tail samples taken for analysis of DNA.

**DNA Analysis.** Mouse tissues were removed and dissected over liquid nitrogen and immediately frozen in liquid nitrogen and stored at -80°C. The general methods used to identify mice carrying the *trans* genes have been previously reported (22, 30). Briefly, DNA was extracted from the tail as described (22, 31), resuspended in 10 mM Tris, pH 7.5, and 1 mM EDTA and blotted onto Gene Screen Plus filter paper under vacuum at concentrations of 2.5, 5, and 10  $\mu$ g using a Schleicher and Schuell slot blot manifold. The number of gene copies per animal was estimated by standardizing each slot blot with known amounts of plasmid DNA containing the *ada* gene. The gene copies per founder are given as a range of values obtained from the founder and F<sub>1</sub> offspring, when available.

For slot blot analysis, the Gene Screen Plus filter was baked for 2 h in a vacuum oven, prehybridized in a solution of 50% formamide, 0.25 M Na<sub>2</sub>HPO<sub>4</sub>, 0.25 M NaCl, 2 mM EDTA, 0.1% dried milk, 7% SDS, and 100  $\mu$ g/ml denatured salmon testes DNA for 2 h at 42°C, and probed with the 1.3-kilobase *Hind*III-*Sma*I *ada* fragment isolated from pSV2*ada*alkB (labeled with [ $\alpha$ - $^{32}$ P]dCTP using the Pharmacia random primer kit). The filters were incubated for 48 h at 42°C, washed in 0.1% standard saline citrate-0.1% SDS at 50°C for 45 min, and exposed to

\* F. Rottman, unpublished results.

x-ray film at  $-70^{\circ}\text{C}$ . To determine the size of the integrated chimeric *trans* gene, 20  $\mu\text{g}$  genomic DNA was digested with either *Xba*I or *Pst*I and *Sph*I at 5 units enzyme/ $\mu\text{g}$  DNA for 16 h at  $37^{\circ}\text{C}$  and separated on a 0.8% agarose gel and analyzed by Southern blotting as previously described, using the conditions described above.

**RNA Analysis.** Animals were killed by cervical dislocation and dissected tissues were immediately frozen in liquid nitrogen and then homogenized in 4 M guanidinium hydrochloride, and the RNA was isolated following cesium chloride centrifugation (31, 32). Total cellular RNA (20  $\mu\text{g}$ ) was separated on a 1% agarose gel containing 0.66 M formaldehyde (33), transferred to Gene Screen Plus, cross-linked to the membrane with UV light for 3 min, and baked for 2 h at  $80^{\circ}\text{C}$ . The membrane was hybridized with the 1.3-kilobase *ada* probe (see above) in buffer consisting of 50% formamide, 1 M NaCl, 0.1%  $\text{NaH}_2\text{PO}_4$ , 0.2% bovine serum albumin, 0.2% Ficoll, 0.1% SDS, and 100  $\mu\text{g}/\text{ml}$  salmon testes DNA. The membranes were hybridized at  $42^{\circ}\text{C}$  for 48 h, washed with  $0.1\times$  NaCl-sodium citrate buffer-0.1% SDS at  $55^{\circ}\text{C}$  for 30 min, and exposed as described above for Southern analysis. Each membrane was washed with 1% glycerol for 3 min at  $80^{\circ}\text{C}$  to remove *ada* probe and rehybridized with GAPDH probe to identify the variability of mRNA loading in each lane.

**Measurement of *O*<sup>6</sup>-Alkylguanine-DNA Alkyltransferase.** Tissues were dissected from animals and frozen in liquid nitrogen. To prepare tissue extract, tissues were suspended at 100 mg/ml in cell extract buffer [70 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.1 mM EDTA, 5% glycerol, and 1 mM dithiothreitol, pH 7.8], homogenized, and sonicated (34). The activity of alkyltransferase in each cell extract was measured as removal of the methyl- $^3\text{H}$  adduct from *O*<sup>6</sup>-[ $^3\text{H}$ ]methylguanine in methyl- $^3\text{H}$  DNA alkylated with *N*-[ $^3\text{H}$ ]methylnitrosourea (4, 34, 35). An alkyltransferase unit of activity is defined as the removal of 1 fmol of *O*<sup>6</sup>-methylguanine from the substrate DNA and is reported as units/ $\mu\text{g}$  cellular DNA (35).

**SDS-PAGE Analysis of Alkyltransferase.** Reaction of the alkyltransferase with substrate [methyl- $^3\text{H}$ ]DNA-containing *O*<sup>6</sup>-[ $^3\text{H}$ ]methylguanine adducts results in covalent transfer of the methyl- $^3\text{H}$  group to the alkyltransferase. Following SDS-PAGE, the two forms of the alkyltransferase can easily be distinguished by molecular weight: the molecular weight of bacterial alkyltransferase is 39,000 with two proteolytic active products ( $M_r = 20,000$  and 19,000) and the molecular weight of the mammalian protein is approximately 23,000 (36). Tissue extract containing 1 mg protein was reacted with 15  $\mu\text{g}$  methyl- $^3\text{H}$  DNA (specific activity 10.4 dpm/fmol *O*<sup>6</sup>-methylguanine) at  $37^{\circ}\text{C}$  for 45 min. The reaction mixture was separated by 11% SDS-PAGE (17) and soaked in Enhance (New England Nuclear) for 1 h at  $22^{\circ}\text{C}$ , and an autoradiogram was exposed.

## RESULTS

**Production of Founder Animals.** The PEPCK *ada* gene (Fig. 1) was injected into the pronuclei of multiple embryos after which 65 normal mice were born. Southern analysis of genomic DNA taken from tail segments at 4 weeks of age identified 6 animals carrying the PEPCK *ada* gene (Fig. 2). A 2.3-kilobase DNA fragment hybridizing to the *ada* probe was identified after digestion with either *Xba*I or with *Sph*I and *Bam*HI. The 2.3-kilobase fragment is the expected size of tandem repeats of the PEPCK *ada trans* gene inserted in a head to tail fashion and should be isolated by *Xba*I digestion (see Fig. 1). The *Sph*I-*Bam*HI digestion should excise the intact *trans* gene of the same size. The presence of a 2.3-kilobase DNA fragment strongly hybridizing to *ada* indicates that the *trans* gene was inserted intact as head to tail concatemers. Other intensively hybridizing bands noted in Fig. 2 indicate that there are other more complex and unique integration patterns of the *trans* gene that also occur in the founder animals. In founders 2, 10, and 21, for instance, some copies of the *trans* gene appear to have lost adjacent *Bam*HI and *Xba*I restriction enzyme sites in the 5' region of the *trans* gene, giving rise to hybridization of the *ada* probe to

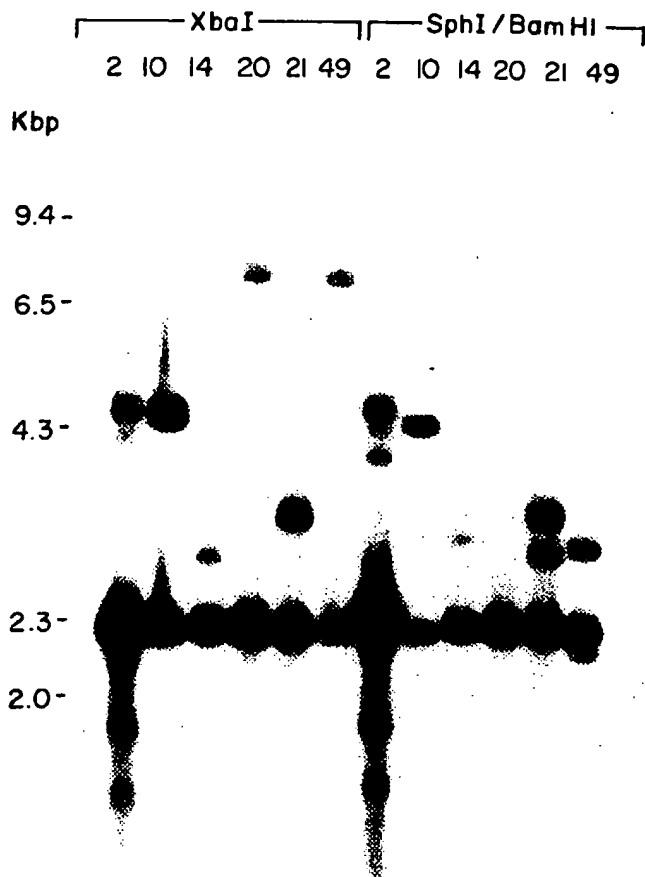


Fig. 2. Identification of the PEPCK *ada trans* gene in founder transgenic mice. Twenty  $\mu\text{g}$  genomic DNA prepared from the tail of founder animals were digested with *Sph*I and *Bam*HI to excise the intact *trans* gene or with *Xba*I which cuts once within the *trans* gene to identify tandem repeats and *trans* gene/genomic DNA junctional fragments. The DNA was separated on a 0.8% agarose gel and subjected to Southern analysis using the intact *ada* gene as probe. The expected size of the *trans* gene is 2.3 kilobase pairs (Kbp).

larger and identical DNA fragments in each instance when genomic DNA was digested with either *Xba*I or *Sph*I and *Bam*HI. In addition to these prominent bands, a faint band is seen in each lane of the group digested with *Xba*I which represents a "junctional fragment" between the *ada* gene and genomic DNA. If there is one insertion locus in each founder, one junctional fragment would be seen. This was, in fact, observed in DNA from each founder. Southern analysis of genomic DNA from the  $F_1$  offspring of founders 10-49 digested with either *Xba*I or *Sph*I/*Bam*HI gave the identical pattern to that seen with the founder animals (data not shown). This also suggests that a single chromosomal insertion locus is present for each founder animal.

Table 1 shows the germ line transmission analysis for each of the founder animals. Founders 10 and 49 have relatively low numbers of gene copies per genome. These founders transmit the *trans* gene to approximately 50% of offspring as predicted if the gene is uniformly present in the germ line. Three founders, 14, 20, and 21, transmit with frequencies of 15-26% and are considered to have the *trans* gene present in a mosaic pattern in the germ line. Finally, founder 2, with more than 100 gene copies/genome, did not transmit the *trans* gene to any of his offspring.

**Analysis of *ada* Gene Expression.** To determine the level and tissue specificity of *ada* gene expression,  $F_1$  offspring of the founder animals were killed, and total cellular RNA was pre-



Table 1 PEPCK *ada* trans gene inheritance pattern by transgenic founders

Six founder animals were analyzed for the number of gene copies/genome by Southern analysis of tail DNA. Outbred F<sub>1</sub> offspring were also analyzed for the transgene by slot blot of tail DNA. Transmission was characterized as heterozygote if approximately 50% of offspring carried the PEPCK *ada* trans gene and mosaic if 10–30% carried the transgene. The *ada* gene was used as probe.

Founder	Gene copies/genome	F <sub>1</sub> transmission, positive/total (%)	Transmission
2 (M)	>100	0/29 (0)	None
10 (M)	5–8	22/38 (58)	Heterozygote
14 (M)	10–30	4/27 (15)	Mosaic
20 (M)	6–9	2/11 (18)	Mosaic
21 (F)	10–30	8/31 (26)	Mosaic
49 (M)	6–9	21/33 (64)	Heterozygote

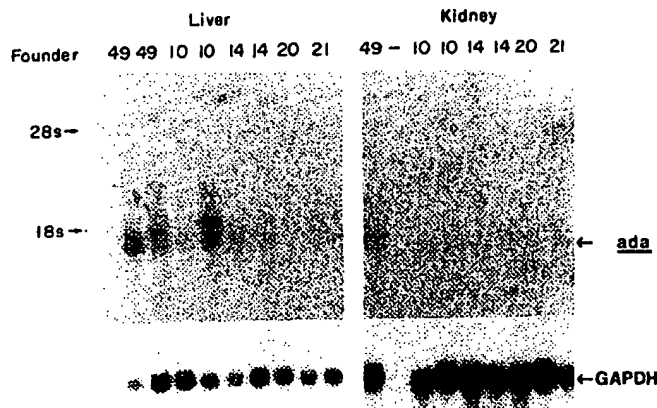


Fig. 3. PEPCK *ada* gene expression in liver and kidney of heterozygote offspring of founder animals. Twenty  $\mu$ g total cellular RNA, prepared from the liver and kidney of F<sub>1</sub> *ada*<sup>+</sup> heterozygote offspring of founder animals, was separated by formaldehyde-agarose gel electrophoresis and subjected to Northern analysis using the intact *ada* gene as probe. As a measure of the variability of mRNA loading in each lane, the membrane was also hybridized with the GAPDH probe as shown. Each lane represents a different animal and is labeled according to the founder parent. —, no mRNA loaded in the lane. A band representing the expected size of the *ada* mRNA transcribed from the PEPCK promoter is seen in each lane. No bands hybridizing with *ada* were seen in RNA isolated from nontransgenic mice (data not shown). The large sized hybridizing signal seen in the kidney of the founder 21 offspring may represent nonspecific hybridization with 28SR RNA and has not been seen in Northern analysis of other founder 21 offspring.

pared from various tissues and analyzed by Northern blot using the 1.3-kilobase *ada* gene as probe. Fig. 3 shows analysis of RNA extracted from the liver and kidney from F<sub>1</sub> offspring of founders 10, 49, 14, 20, and 21. The amount of mRNA containing *ada* was much higher in the liver than kidney, whereas the level of endogenous PEPCK expression was similar in both tissues (data not shown). No RNA hybridizing with *ada* was observed in the lung or spleen (data not shown). There was some variation in the level of *ada* mRNA expression among F<sub>1</sub> offspring from different founders. However, all animals derived from the 5 founders expressed *ada* mRNA in the liver and kidney. The sex and age of the animals also did not seem to affect *ada* gene expression (data not shown).

**Tissue Alkyltransferase Activity in Animals Carrying the PEPCK *ada* trans Gene.** Fig. 4 shows the alkyltransferase activity in the liver and kidney in control animals (C) and outbred F<sub>1</sub> offspring from each of the transmitting founder animals. Alkyltransferase activity was measured as removal of O<sup>6</sup>-methylguanine adducts from substrate [methyl-<sup>3</sup>H]DNA, methylated with N-[<sup>3</sup>H]nitroso-N-methylurea (32). Thus, activity in transgenic compared to control animals can be compared on the basis of relative capacity for removal of O<sup>6</sup>-methylguanine DNA adducts. While the bacterial alkyltransferase will also remove O<sup>6</sup>-methylthymine adducts and methylphosphotriester

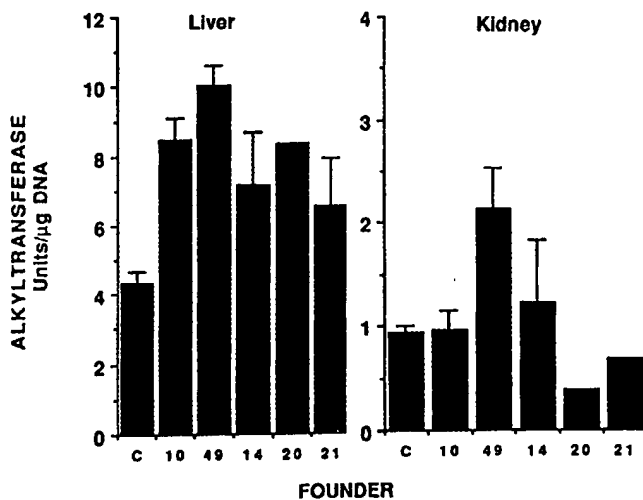


Fig. 4. Alkyltransferase in tissue of transgenic mice carrying PEPCK *ada*. Liver and kidney tissue extracts from nontransgenic control (C) and F<sub>1</sub> offspring of founder animals 10, 49, 14, 20, and 21 were assayed for alkyltransferase activity as described in "Materials and Methods." Bar, mean  $\pm$  SD of the average of duplicate determinations of activity in 3–7 different animals except for founder 20 offspring in which only 2 animals were available.

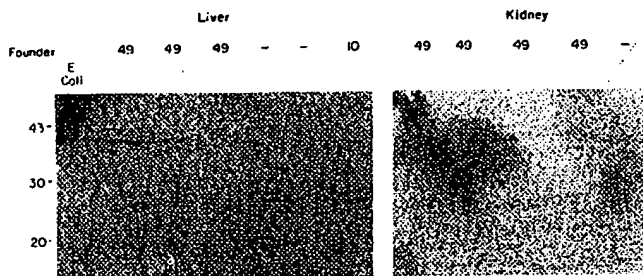


Fig. 5. Identification of bacterial alkyltransferase in transgenic mice. Cell extracts from liver and kidney containing 1 mg protein were reacted with 15  $\mu$ g methyl-<sup>3</sup>H DNA for 45 min at 37°C and the proteins separated by SDS-PAGE. An autoradiogram was exposed. The endogenous alkyltransferase has a molecular weight of approximately 23,000. A characteristic doublet or triplet (M<sub>r</sub> = 22,000–26,000) was always observed in liver extracts. Alkyltransferase derived from the bacterial *ada* gene has a molecular weight of 39,000. 10 and 49, founders from which F<sub>1</sub> offspring were derived; —, nontransgenic mice; numbers on left, M<sub>r</sub>  $\times$  1000 of marker proteins.

adducts (37), these are not measured in the enzyme assay we performed (34). Thus, a doubling in activity in transgenic *versus* control liver would indicate an equal number of alkyltransferase molecules of mammalian and bacterial origin. Each group of F<sub>1</sub> offspring had a 1.5–2.4-fold increase in alkyltransferase activity in the liver as compared to control animals. The levels of kidney alkyltransferase activity are much lower than in the liver, and F<sub>1</sub> offspring from founders 10, 14, 20, and 21 did not show increased renal alkyltransferase activity compared to nontransgenic litter mates. However, renal alkyltransferase activity was increased in offspring from founder 49; [2.1  $\pm$  0.3 (mean  $\pm$  SE) *versus* 0.9  $\pm$  0.1 units/ $\mu$ g DNA]. Reduced renal alkyltransferase activity in founder 20 offspring may be an artifact of the small sample size ( $n$  = 2).

To confirm that the increased alkyltransferase was due to translation from *ada* mRNA, cell extracts were incubated with [methyl-<sup>3</sup>H]DNA-containing O<sup>6</sup>-[<sup>3</sup>H]methylguanine DNA adducts to label functionally active alkyltransferase molecules of both bacterial and mammalian origin, and then the proteins were separated by SDS-PAGE. Fig. 5 shows that liver and kidney from transgenic animals contained a labeled protein of M<sub>r</sub> 39,000, which was the expected size of the bacterial alkyltransferase protein transcribed from the *ada* mRNA. The bac-



terial alkyltransferase protein band was much stronger in liver extracts than in kidney extracts. Even though renal alkyltransferase activity was not increased in offspring of founder 10, a faint labeled protein of  $M_r$  39,000 was seen, indicating that these animals also expressed the bacterial alkyltransferase in the kidney (data not shown). The amount of mammalian alkyltransferase found in the liver and kidney was the same in both transgenic animals and their nontransgenic litter mates. The molecular weight of mammalian alkyltransferase is about 23,000 (36) and multiple bands were always seen in the 22,000–26,000 molecular weight region in the liver, possibly due to the action of a nonspecific protease. In the kidney, the mammalian alkyltransferase was expressed at low levels and could only faintly be detected by SDS-PAGE.

**Dietary Regulation of PEPCK *ada* Expression.** The activity of hepatic PEPCK is induced by a diet high in protein but low in carbohydrate and markedly reduced by a diet high in carbohydrate (38). To test the regulation of the *trans* gene, F<sub>1</sub> offspring of founders 10 and 49 and nontransgenic litter mates were fed either regular rodent chow or a diet high in protein or high in carbohydrate for 1 or 4 weeks prior to being killed. There was a marked increase in *ada* mRNA in the liver of animals fed the high protein diet compared to transgenic animals fed a regular diet for 1 week (Fig. 6). We could detect no *ada* mRNA in animals fed the carbohydrate diet for the same period. Expression of the *ada* gene in the kidney was induced in 1 of 3 founder 10 offspring and 1 of 3 founder 49 offspring who were fed a diet high in protein (Fig. 7). However, PEPCK *ada* expression in the kidney was not reduced by a diet high in carbohydrate (data not shown). These results are consistent with the pattern of regulation of expression of the endogenous PEPCK gene in the liver and kidney (21, 24, 25, 39). Transgenic mice fed the high protein diet had a further increase in hepatic and renal alkyltransferase activity above that of nontransgenic (*ada*<sup>-</sup>) and uninduced (*ada*<sup>+</sup>) transgenic animals, whereas animals fed the carbohydrate diet had alkyltransferase activity in the liver and kidney that was similar to that seen in nontransgenic animals (Fig. 8). The induction of kidney alkyltransferase by a diet high

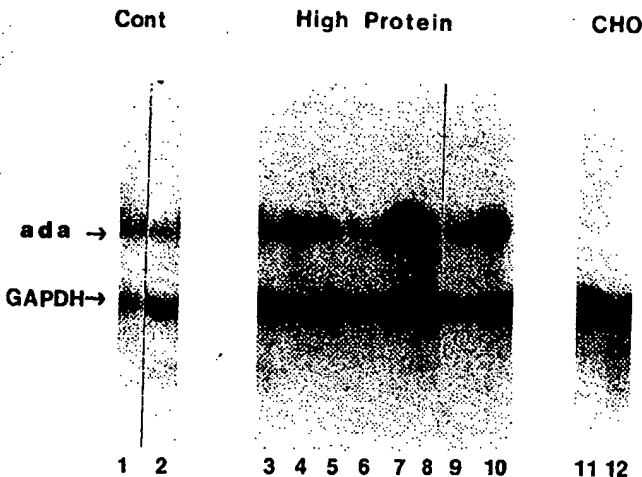


Fig. 6. Dietary regulation of PEPCK *ada* in the liver of transgenic animals. Transgenic mice were fed a regular diet (Cont, Lanes 1 and 2). A high protein diet (High Protein, Lanes 3–10) or a high carbohydrate diet (CHO, Lanes 11 and 12) for 7 days and 20  $\mu$ g total cellular RNA extracted from liver was subjected to Northern analysis using the *ada* probe. The variability of mRNA loading is indicated by the intensity of the signal produced after probing the same membrane with GAPDH. GAPDH mRNA levels did not change during dietary manipulation. Increased *ada* expression was seen in liver from the animals on the high protein diet and decreased or absent expression was seen in animals on a carbohydrate diet. RNA isolated from offspring of founder 49 are shown in Lanes 1–4 and 9–12 and from founder 10 in Lanes 5–8.

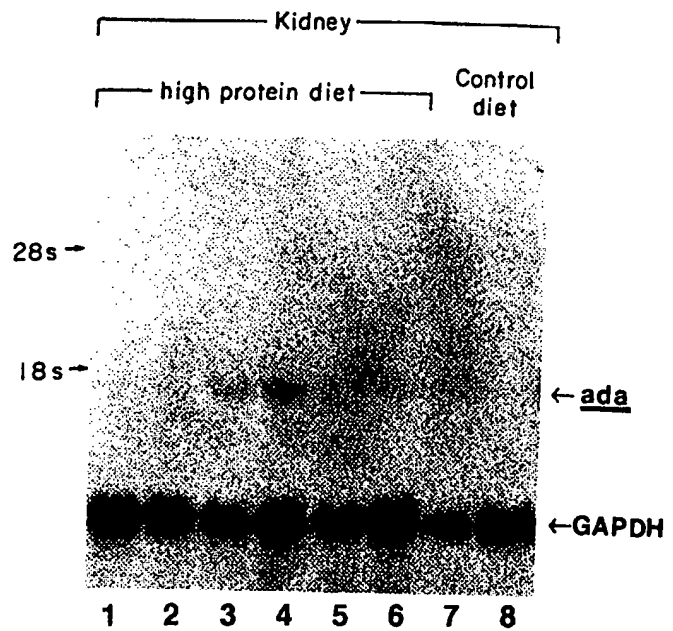


Fig. 7. Induction of PEPCK *ada* in kidney of transgenic animals fed a high protein diet. Transgenic animals were fed either a regular (control) or high protein diet for 7 days, and 20  $\mu$ g total cellular RNA extracted from kidney was analyzed by Northern analysis using, sequentially, *ada* and GAPDH as probes. Increased expression of *ada* was seen in kidney of two animals fed the diet high in protein. Lanes 1–3, RNA from offspring of founder 10; Lanes 4–8, RNA from offspring of founder 49. The level of *ada* mRNA in kidney of a control diet was greater in offspring of founder 49 than founder 10 (see Fig. 3).

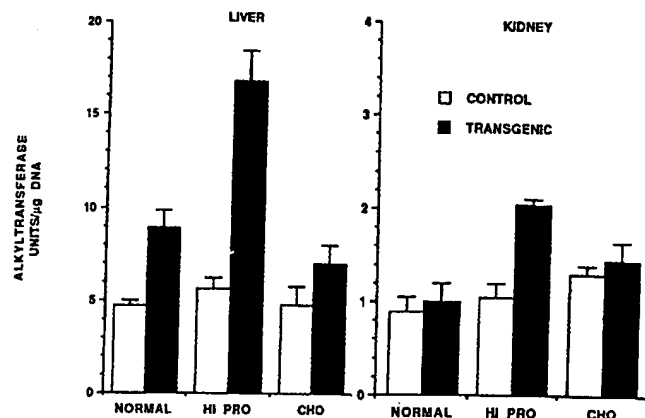


Fig. 8. Dietary regulation of liver and kidney alkyltransferase activity. Transgenic animals fed a regular (NORMAL), high protein (HI PRO), or high carbohydrate diet (CHO) for 7 days were sacrificed and liver and kidney were assayed for alkyltransferase. White columns, control nontransgenic *ada*<sup>-</sup> litter mates; black columns, *ada*<sup>+</sup> transgenic mice offspring from founders 10 and 49. Data represent the means  $\pm$  SE (bars) of duplicate determinations of the activity in tissues derived from 3–5 mice in each group.

in protein was greater in offspring of founder 49 than in offspring from the other founder animals. Hepatic alkyltransferase activity in transgenic animals fed a high protein diet for 1 week increased to  $16.6 \pm 1.5$  units/ $\mu$ g DNA compared to  $5.3 \pm 0.6$  units/ $\mu$ g DNA in the nontransgenic (*ada*<sup>-</sup>) litter mates fed a high protein diet and  $7.0 \pm 0.8$  units/ $\mu$ g DNA in carbohydrate-fed transgenic mice.

During these studies, we noted that there was no absolute correlation between *ada* mRNA levels and alkyltransferase activity, particularly comparing animals across founder lines. Within the same founder and the same tissue, there was a relatively good correlation between *ada* mRNA and alkyltransferase activity. Animals fed a high protein diet for up to 1 month showed similar increases in hepatic and renal alkyltrans-

ferase activity as that seen at 1 week. SDS-PAGE analysis of functional alkyltransferase (Fig. 9) shows that the increase in liver alkyltransferase in the transgenic animals fed a high protein diet was entirely due to bacterial alkyltransferase expressed in the mammalian tissues. Little or no bacterial alkyltransferase was detected in mice fed the diet high in carbohydrates. The resolution of the SDS-PAGE for kidney extracts was not sufficient to identify a change in alkyltransferase in animals fed a high protein diet (data not shown).

## DISCUSSION

Our studies show that transgenic animals carrying the chimeric PEPCK *ada* gene have tissue-specific and -regulatable *ada* expression and appropriately increased alkyltransferase activity. These studies indicate that functional alkyltransferase of bacterial origin can be produced and expressed in transgenic animals. The transgenic animals we have characterized may provide an animal model for evaluating the impact of increased tissue alkyltransferase on *N*-nitroso toxicity in the liver and kidney. By targeting *ada* gene expression to liver and kidney, we have increased the alkyltransferase in these two tissues rather than in all tissues. The liver alkyltransferase activity obtained during induction with a high protein diet for 1 week ( $16.6 \pm 1.5$  units/ $\mu$ g DNA) is very high relative to that in other mouse tissues [range, 0.15–2.6 units/ $\mu$ g DNA (35, 39, 40)] and higher than that in rat tissues (range, 0–6.8 units/ $\mu$ g DNA) or human tissues [range 3.1–15 units/ $\mu$ g DNA (34, 35)] with the exception of human liver which has alkyltransferase activity of  $55 \pm 9.7$  units/ $\mu$ g DNA (4, 34, 35). Thus, on a relative basis, the mouse liver of these transgenic animals should now be much more resistant to DNA damage induced by *N*-nitroso compounds than normal, nontransgenic mice of the same strain.

In the mouse, tissue alkyltransferase activity is lower than in

other mammalian species such as human, rat, or monkey (35, 36, 40). This may be one reason that the mouse is susceptible to the toxicity and carcinogenicity of *N*-nitroso compounds (1, 2, 12, 41). It remains to be tested whether increased alkyltransferase activity will alter the toxicity profile in normal tissues of a number of *N*-nitroso compounds both in terms of acute cytotoxicity, DNA adduct formation, and overall carcinogenicity. A recent study has documented that expression of *ada* in a human tumor cell xenograft resulted in increased nitrosourea resistance (42). Their study was the first to document that expression of the bacterial *ada* gene will increase resistance to *N*-nitroso compounds in mammalian cells *in vivo*.

Although we have measured removal of *O*<sup>6</sup>-methylguanine from methylated DNA as an index of bacterial alkyltransferase expression, it should be noted that the bacterial protein differs from the mammalian alkyltransferase because it is also able to remove *O*<sup>4</sup>-alkylthymine and alkylphosphotriester DNA adducts (43, 44). These adducts are formed by different *N*-nitroso compounds to varying extents relative to *O*<sup>6</sup>-alkylguanine (10, 45), but the repair of these adducts by mammalian enzymes has not been extensively defined (43). However, the persistence of *O*<sup>4</sup>-alkylthymine is associated with carcinogenicity (45, 46). Thus, it is possible that increased repair of these adducts, particularly *O*<sup>4</sup>-alkylthymine, will influence carcinogenicity studies. Others have documented the breakdown of the intact bacterial alkyltransferase (*M*, 39,000) to two functionally active fragments (*M*, 19,000 and 20,000) in mammalian cells following transfection (17, 19, 47). This cleavage is thought to be due to a site sensitive to a thiol protease (48). While these cleaved fragments can function in mammalian cells, they were detected only in very low levels *in vivo* in our transgenic animals, indicating that most of the bacterial protein remains intact. If mammalian liver alkyltransferase sensitivity to a nonspecific protease is the explanation for the triplet band seen on SDS-PAGE (Figs. 5 and 8), it is of interest that this proposed protease does not affect the bacterial alkyltransferase. Addition of protease inhibitors does not alter total alkyltransferase activity/mg protein<sup>5</sup> but does seem to aid in the purification of the hepatic alkyltransferase (36).

The endogenous PEPCK gene is expressed in a highly regulated manner in the liver where it is inducible during gluconeogenesis and in the kidney where it is induced by acidosis (21–24). Previous studies with the chimeric PEPCK-bovine growth hormone gene in transgenic animals found the gene to be inducible in the liver by a high protein diet and inhibited by a diet high in carbohydrate (22). We have also found that the tissue specificity of PEPCK *ada* gene expression was retained when a truncated fragment of the 5'-flanking region of PEPCK was used. It is also possible that the 5th exon of the bovine growth hormone gene present in the PEPCK *ada* trans gene contributed to the stability of gene expression (for a review of factors affecting *trans* gene expression see Ref. 48).

While two other groups have published preliminary reports describing transgenic animals containing the metallothionein promoter linked to *ada* (50, 51), there are a number of advantages of the PEPCK promoter that make it useful in this system. Most importantly, the PEPCK promoter is inducible by dietary manipulations, whereas the metallothionein promoter requires the use of heavy metals, which are known to inhibit the alkyltransferase (52). Second, PEPCK *trans* genes can be turned off by a diet high in carbohydrate, whereas there is often a high level of basal expression from other promoters used in trans-

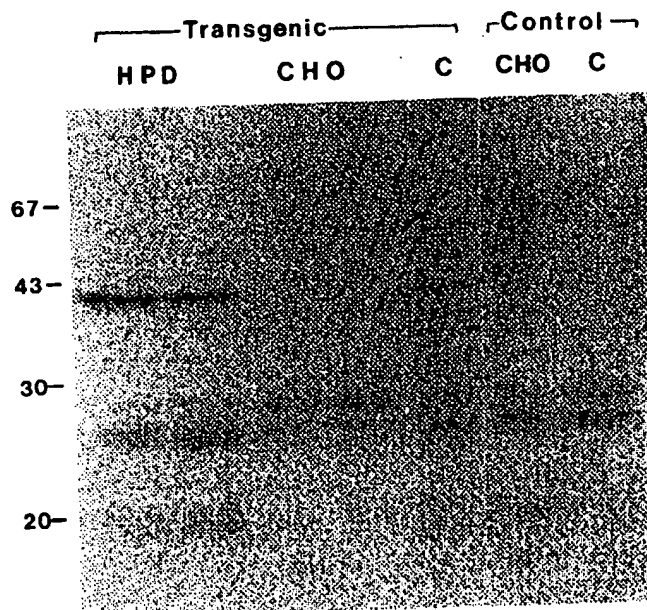


Fig. 9. Induction of bacterial alkyltransferase by a high protein diet in livers of transgenic animals. Liver tissue extract containing 1 mg protein was reacted with <sup>3</sup>H-DNA and separated by SDS-PAGE as described in Fig. 4. The increase in alkyltransferase activity seen in livers of animals fed a high protein diet was confined to the band of molecular weight 39,000 which is the size of the bacterial alkyltransferase protein coded for by *ada*. Note the absence of the protein of molecular weight 39,000 in the animals fed a high carbohydrate diet. Transgenic, offspring of founder 49; control, nontransgenic mice; numbers on left, *M*,  $\times 1,000$  marker proteins. HPD, high protein diet; CHO, high carbohydrate diet; C, control or regular diet.

<sup>5</sup> S. L. Gerson, unpublished results.

genic animal work (49). In our studies of PEPCK *ada*, we found that neither the diet high in protein or carbohydrate altered endogenous mammalian alkyltransferase activity so that the change in the total alkyltransferase activity was due entirely to the bacterial protein. Third, McGrane *et al.* (53) have found that a PEPCK *trans* gene begins to be expressed at birth so that heterologous gene expression will not adversely affect animal development *in utero* (49, 54).

The ability to regulate expression of *ada* by transient dietary manipulation and thus to alter the liver alkyltransferase 3–4-fold may be important in the design of future carcinogenicity and DNA repair studies using *N*-nitroso compounds. Turning off expression of the bacterial alkyltransferase could be an important control in these experiments. Conversely, turning on the *ada* gene at various times after carcinogen exposure will help define the time course of DNA damage and repair as it relates to carcinogenicity. The use of *O*<sup>6</sup>-methylguanine to deplete tissue alkyltransferase *in vivo* (55) is another possible modulator for these studies. Future carcinogenicity studies will be facilitated by the consistent pattern of PEPCK *ada* expression observed in the liver of F<sub>1</sub> offspring from multiple founder animals and in the kidney of founder 49 offspring. Understanding the role of a single DNA repair protein in tissue-specific carcinogenicity in these transgenic animals will help elucidate some of the basic mechanisms of tumor induction.

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# Site-Directed Mutagenesis by Gene Targeting in Mouse Embryo-Derived Stem Cells

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## Summary

We mutated, by gene targeting, the endogenous hypoxanthine phosphoribosyl transferase (HPRT) gene in mouse embryo-derived stem (ES) cells. A specialized construct of the neomycin resistance (*neo'*) gene was introduced into an exon of a cloned fragment of the *Hprt* gene and used to transfect ES cells. Among the G418<sup>r</sup> colonies, 1/1000 were also resistant to the base analog 6-thioguanine (6-TG). The G418<sup>r</sup>, 6-TG<sup>r</sup> cells were all shown to be *Hprt*<sup>-</sup> as the result of homologous recombination with the exogenous, *neo'*-containing, *Hprt* sequences. We have compared the gene-targeting efficiencies of two classes of *neo'*-*Hprt* recombinant vectors: those that replace the endogenous sequence with the exogenous sequence and those that insert the exogenous sequence into the endogenous sequence. The targeting efficiencies of both classes of vectors are strongly dependent upon the extent of homology between exogenous and endogenous sequences. The protocol described herein should be useful for targeting mutations into any gene.

## Introduction

Gene targeting—the homologous recombination of DNA sequences residing in the chromosome with newly introduced DNA sequences—provides a means for systematically altering the mammalian genome (Smithies et al., 1985; Thomas et al., 1986; Thomas and Capecchi, 1986). A desired alteration would first be introduced into a cloned DNA sequence, and gene targeting would then transfer the alteration into the genome. Gene targeting should be equally effective for correcting or mutating the desired chromosomal locus.

We initiated our analysis of gene targeting in cultured mammalian cells by studying recombination between a defective gene residing in the chromosome and newly introduced plasmid DNA carrying a different mutation in the same gene. For those experiments, we first established cell lines containing a mutant neomycin resistance gene (*neo'*) integrated into the genome of mouse L cells. We were then able to restore the gene via homologous recombination by injecting DNA carrying a different mutation in the *neo'* gene. In the course of these experiments we uncovered two mechanisms for altering chromosomal sequences. The first involved the transfer of information, by homologous recombination, from the newly introduced DNA into the cognate chromosomal sequence (Thomas et al., 1986). The second involved inducing mutations in the

homologous chromosomal sequence by what appears to entail incorrect repair of a heteroduplex formed between the newly introduced DNA and the cognate chromosomal sequence (Thomas and Capecchi, 1986). Each of the two methods has its own advantages. The transfer of information by homologous recombination allows one to mutate or correct the desired chromosomal locus in a defined manner. On the other hand, the frequency of altering chromosomal sequences by heteroduplex-induced mutagenesis promises to be higher than via homologous recombination. This could prove to be a useful method for generating a large number of random mutations in specific genes.

In this current study we have extended our analysis of gene targeting by using an endogenous gene as the target and by using embryo-derived stem (ES) cells as the recipient cell line.

The target gene is hypoxanthine phosphoribosyl transferase (*Hprt*). This gene was selected primarily for two reasons. First, the *Hprt* gene lies on the X-chromosome. Since ES cells derived from male embryos are hemizygous for *Hprt*, only a single copy of the *Hprt* gene needs to be inactivated in order to yield a selectable phenotype. Second, selection procedures have been developed for isolating *Hprt*<sup>-</sup> mutants. By far the most common pathway for cells in culture to become resistant to the base analog 6-thioguanine (6-TG) is to acquire a mutation in the *Hprt* gene (Sharp et al., 1973; Wahl et al., 1975).

ES cells were chosen for these experiments because, following inactivation of a chosen gene by gene targeting, they should provide the means to generate mice with the desired mutation. ES cells have been shown to be pluripotent in vitro and in vivo (Evans and Kaufman, 1981; Martin, 1981). When reintroduced into mouse blastocysts, these cells contribute efficiently to the formation of chimeras, including contributions to a functional germ line (Bradley et al., 1984). In addition, it has been shown recently that these cells can be manipulated in vitro without losing their capacity to generate germ-line chimeras. Following transfection with the *neo'* gene and selection for G418<sup>r</sup>, these ES cells were used to produce germ-line chimeras that stably transmitted G418<sup>r</sup> to subsequent generations (Gossler et al., 1986; Robertson et al., 1986). HPRT-deficient mice were produced from ES cells that were either selected for spontaneous *Hprt*<sup>-</sup> mutations (Hooper et al., 1987) or selected for *Hprt*<sup>-</sup> following the random insertion of retroviral DNA into the mouse genome (Kuehn et al., 1987).

Here we describe the site-directed inactivation of the endogenous *Hprt* gene in male ES cells by gene targeting. We examine some parameters that affect the gene-targeting frequency as well as the mechanism of gene inactivation mediated by different recombinant vectors. Under optimal conditions, we find that 1/1000 cells transformed by exogenous DNA can undergo a gene-targeting event. The advantage of inactivating specific genes via gene targeting compared with random mutagenic methods such

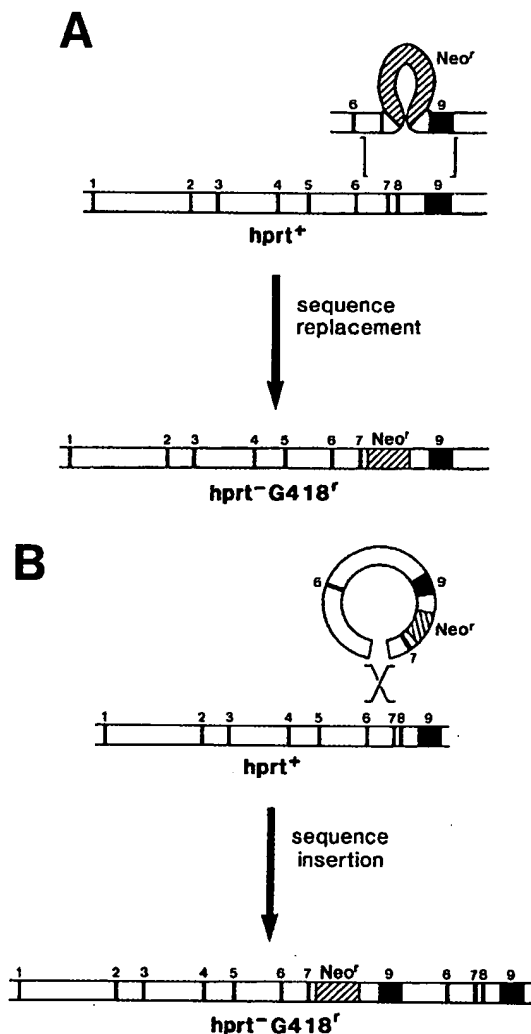


Figure 1. Disruption of the *Hprt* gene by Gene Targeting

Two schemes for gene disruption, one by sequence replacement vectors and one by sequence insertion vectors, are depicted. Vectors of both classes contain *Hprt* sequences interrupted in the eighth exon with the *neo<sup>r</sup>* gene.

(A) Sequence replacement. Sequence replacement vectors are designed such that upon linearization, the vector *Hprt* sequences remain colinear with the endogenous sequences. Following homologous pairing between vector and genomic sequences, a recombination event replaces the genomic sequences with the vector sequences containing the *neo<sup>r</sup>* gene.

(B) Sequence insertion. Sequence insertion vectors are designed such that the ends of the linearized vector lie adjacent to one another on the *Hprt* map. Pairing of these vectors with their genomic homolog, followed by recombination at the double strand break, results in the entire vector being inserted into the endogenous gene. This produces a duplication of a portion of the *Hprt* gene. Open boxes indicate introns; closed boxes indicate exons, numbered according to the map of Melton et al. (1984); the crosshatched box indicates the *neo<sup>r</sup>* gene.

as chemical mutagenesis or retroviral DNA insertion is twofold. First, the nature of the mutant allele is at the discretion of the experimenter, and second, unlike random mutagenic events, the frequency of the targeting events is sufficiently high to make the procedure applicable to non-selectable genes.

## Results

The *Hprt* gene encompasses over 33 kb of DNA and contains 9 exons that encode 1307 nucleotides of mRNA (Melton et al., 1984). In Figure 1 we illustrate our strategies for inactivating the *Hprt* gene. The eighth exon in a cloned fragment of *Hprt* is disrupted by inserting the *neo<sup>r</sup>* gene. Following introduction of this DNA into ES cells, homologous recombination transfers this disruption into the endogenous *Hprt* gene, rendering the cells *neo<sup>r</sup>-Hprt<sup>-</sup>* and therefore resistant to the drug G418 and the base analog 6-TG.

Using gene targeting in yeast as a paradigm (Hinnen et al., 1978; Orr-Weaver et al., 1981), we constructed two classes of vectors that we believed would disrupt the *Hprt* gene either by replacing endogenous sequences or by inserting into the endogenous sequences. We termed these recombinant *neo<sup>r</sup>-Hprt* vectors replacement vectors (RV) and insertion vectors (IV). The mechanism of inactivating the endogenous *Hprt* gene by these two vectors is depicted in Figures 1A and 1B. It was of interest to determine whether one or the other class of vectors was more efficient at targeting. Furthermore, since the end results using these two classes of vectors were predicted to be different (note the partial duplication of the gene in Figure 1B), each could be used to generate different mutant alleles.

### Reengineering the *neo<sup>r</sup>* Gene

In the schemes outlined in Figure 1 for site-specific mutagenesis of the *Hprt* gene, the *neo<sup>r</sup>* gene is used both to disrupt the coding sequence of the target gene and as a tag to monitor the integration of the newly introduced DNA into the recipient genome. Effective use of the *neo<sup>r</sup>* gene as a tag requires expression of the gene in the *Hprt* locus. In general, if the *neo<sup>r</sup>* gene is to be used in a similar fashion to inactivate other genes, it must be expressed in as many chromosomal sites as possible.

In one of our mutagenesis schemes (Figure 1A), we require the newly added *neo<sup>r</sup>*-containing sequences to convert the endogenous gene. We suspect that the frequency of gene conversion at the target locus may be inversely proportional to the length of nonhomology in the converting sequence. This certainly appears to be the case for intrachromosomal gene conversion (Letsou and Liskay, 1987).

Keeping the above points in mind, we have redesigned the *neo<sup>r</sup>* gene to optimize expression in ES cells while maintaining its size at a minimum. In Figure 2 we illustrate the *neo<sup>r</sup>* gene we have modified for this purpose. It is designated pMC1Neo. The neomycin protein coding sequence (d) is from the bacterial transposon Tn5. The promoter (b) that drives the *neo<sup>r</sup>* gene is derived from the herpes simplex virus thymidine kinase gene (HSV-*tk*). This promoter appears to be effective in embryonal carcinoma (EC) cells (Nicolas and Berg, 1983; Rubenstein et al., 1984; Stewart et al., 1985). To increase the efficiency of the *tk* promoter, we introduced a duplication of a synthetic 65 bp fragment (a) derived from the PyF441 polyoma virus enhancer. This fragment encompasses the



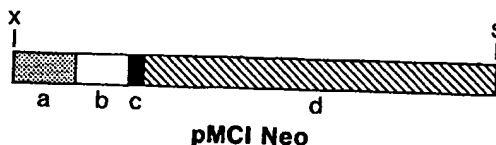


Figure 2. The *neo'* Gene from pMC1Neo

The structural gene and its control elements are contained on a 1 kb cassette flanked by an XhoI site (X) and a Sall site (S) in a pUC derivative plasmid. (a) A tandem repeat of the enhancer region from the polyoma mutant PYF441 consisting of bases 5210–5274 (Fujimura et al., 1981). (b) The promoter of HSV-tk, from bases 92–218 (McKnight, 1980). (c) A synthetic translation initiation sequence, GCCAATATGGGATCGGCC. (d) The *neo'* structural gene from Tn5, including bases 1555–2347 (Beck et al., 1982).

DNA sequence change that allows the polyoma mutant to productively infect EC cells (Linney and Donerly, 1983). Finally, because the native *neo'* gene translation initiation signal is particularly unfavorable for mammalian translation, a synthetic sequence (c) was substituted using Kozak's rules as a guide (Kozak, 1986). A series of transfection experiments (data not shown) demonstrated that pMC1Neo, inserted into the eighth exon of *Hprt*, could utilize the *Hprt* poly(A) addition signal. This obviated the need to include a poly(A) addition signal in the construction.

Each of the above modifications was found to contribute additively to the transfection efficiency. The contribution of each change was assessed by introducing the different *neo'* constructs into mouse fibroblasts (L cells) and mouse ES cells either by microinjection (Capecchi, 1980) or by electroporation and assaying for the yield of G418<sup>r</sup> colonies (data not shown).

In Figure 3 we illustrate parallel experiments comparing the transfection efficiency of three *neo'* vectors, pRSVNeo, pSV2Neo, and pMC1Neo, in ES cells. The DNA was introduced by electroporation. pRSVNeo contains the *neo'* gene driven by the long terminal repeat from the avian Rous sarcoma virus (Hudziak et al., 1982). This promoter, with its accompanying enhancer, functions very efficiently in mouse fibroblasts (Luciw et al., 1983) but is seen here

to function poorly in ES cells. pSV2Neo is an SV40 promoter-enhancer-based vector (Southern and Berg, 1982) that appears to function moderately well in ES cells. From Figure 3 it is apparent that pMC1Neo not only yields more G418<sup>r</sup> colonies than either pRSVNeo or pSV2Neo, but also that the colonies are larger (i.e., the cells grow faster). In mouse fibroblasts, all three vectors yield G418<sup>r</sup> colonies at comparable efficiencies.

In Table 1 the transfection efficiencies of pRSVNeo, pSV2Neo, and pMC1Neo are quantitatively compared. From these data, it is apparent that pMC1Neo yields 300- to 800-fold and 25- to 50-fold more G418<sup>r</sup> colonies than pRSVNeo or pSV2Neo, respectively. One interpretation of these results is that, since these vectors are inserting randomly into the mouse genome, the transfection efficiencies reflect the relative number of integration sites within the genome that are compatible with sufficient *neo'* gene expression to yield G418<sup>r</sup> colonies. The higher transfection efficiency of pMC1Neo may prove critical when attempting to use *neo'* as a tag for targeting into genes that are either expressed at low levels, such as *Hprt*, or not at all. We have never observed G418<sup>r</sup> colonies following mock transfections of ES cells (see Table 1).

#### Electroporation

We have used electroporation to introduce the *neo'*-*Hprt* recombinant vectors into ES cells. The conditions for electroporation (Neumann et al., 1982; Potter et al., 1984; Chu et al., 1987) are described in Experimental Procedures. Under our optimal transfection conditions, 40%–60% of the cells survived electroporation and approximately 1/1000 surviving ES cells became G418<sup>r</sup>. The conditions for electroporation were further chosen to yield predominantly single copy integrants. Of a dozen G418<sup>r</sup> cell lines analyzed by Southern transfer, each contained a single copy of pMC1Neo integrated in the mouse genome (data not shown).

#### Recombinant Vectors

In Figure 4, we illustrate the vectors used to inactivate the *Hprt* gene. The vectors contain sequences from the 3' portion of the mouse *Hprt* gene cloned into a pUC9 plasmid.

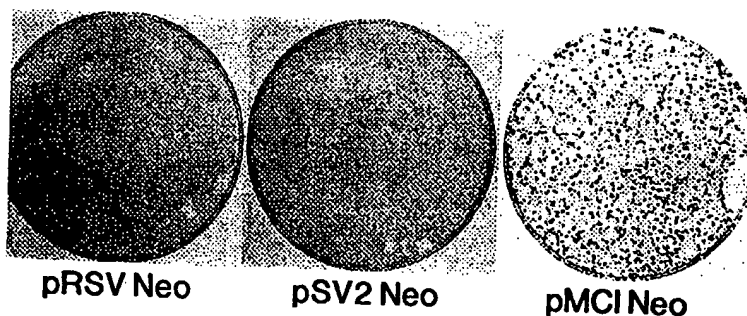


Figure 3. G418<sup>r</sup> ES Cells Obtained by Transfection with pRSVNeo, pSV2Neo, or pMC1Neo  
ES cells were transfected by electroporation with 25 µg/ml of the respective, linearized recombinant *neo'* vector. The conditions for electroporation, cell culture, and selection for G418<sup>r</sup> colonies are described in Experimental Procedures. Ten days following electroporation, the cells were fixed with cold methanol and stained with Giemsa. pMC1Neo does not contain its own poly(A) addition signal (see text). To evaluate its transfection efficiency, either a synthetic poly(A) addition signal derived from HSV-tk, nucleotides 1481–1530 (McKnight, 1980; Zhang et al., 1986), or a fragment from the mouse *Hprt* gene (ScaI site in exon 8 to the BglII site 3' to the end of the gene) was added.

Table 1. Efficiency of Transfection

Vector	Exp.	No. of Cells Surviving Electroporation	No. of G418 <sup>r</sup> Colonies	Frequency of G418 <sup>r</sup> Colonies
0	1	$9.0 \times 10^7$	0	0
	2	$9.4 \times 10^7$	0	0
pRSVNeo	1	$8.5 \times 10^7$	$1.2 \times 10^2$	$1.4 \times 10^{-6}$
	2	$6.3 \times 10^7$	$2.0 \times 10^2$	$3.2 \times 10^{-6}$
pSV2Neo	1	$8.7 \times 10^7$	$2.1 \times 10^3$	$2.4 \times 10^{-5}$
	2	$9.3 \times 10^7$	$4.2 \times 10^3$	$4.5 \times 10^{-5}$
pMC1Neo	1	$6.0 \times 10^7$	$7.5 \times 10^4$	$1.25 \times 10^{-3}$
	2	$7.2 \times 10^7$	$6.8 \times 10^4$	$0.94 \times 10^{-3}$

ES cells were transfected by electroporation with 25  $\mu$ g/ml of either linearized pRSVNeo, pSV2Neo, or pMC1Neo. The conditions for electroporation, cell culture, and selection for G418<sup>r</sup> colonies are described in Experimental Procedures.

In all vectors, the 1 kb *neo<sup>r</sup>* cassette from pMC1Neo has been inserted into the eighth exon of the *Hprt* gene. To minimize the extent of nonhomology between the endogenous *Hprt* gene and the newly introduced DNA, sequences required for growth of the recombinant vector in bacteria were removed prior to introduction into ES cells. In the process of removing these sequences, the recombinant vector is converted to linear DNA that, compared with supercoiled DNA, is a better substrate for gene targeting (Thomas et al., 1986). As discussed previously, these vectors fall into two classes, sequence replacement vectors and sequence insertion vectors, based on the predicted mode of targeting (see Figure 1).

Sequence replacement vectors were designed such that upon linearization, the vector *Hprt* sequences would remain colinear with the endogenous *Hprt* sequences. In other words, the 5' and 3' ends of the vector would correspond to the 5' and 3' extents of sequence homology with the endogenous gene (see Figure 1A). Three different sequence replacement vectors were used in this study. All of them contain a common 3' endpoint, 2.8 kb downstream from the site of the *neo<sup>r</sup>* gene insertion, but differ in the length of *Hprt* sequences 5' from the insertion. The total length of *Hprt* homology in vectors pRV4.0; pRV5.4, and pRV9.1, is 4.0, 5.4, and 9.1 kb, respectively.

The alternate class of vectors, the sequence insertion vectors, were designed such that the separation of the *Hprt* vector from the pUC9 plasmids concomitantly creates a double strand break within the *Hprt* sequences. The 5' and 3' ends of these vectors thus lie adjacent to one another along the *Hprt* map (see Figure 1B). Two sequence insertion vectors were used in these experiments, pIV3.7 and pIV9.3. The endpoints of both linearized vectors arise from the BstEII site 1.1 kb upstream from the site of the *neo* insertion in exon 8. Both vectors contain the same 1.2 kb of *Hprt* sequences 3' from the *neo* insertion, but differ in the length of homology at the 5' side of the *neo* insertion.

#### Targeting with Sequence Replacement Vectors

DNA from the three sequence replacement vectors were linearized to create the substrates shown in Figure 4 and introduced into ES cells by electroporation. Aliquots of these cells were subjected to one of three growth condi-

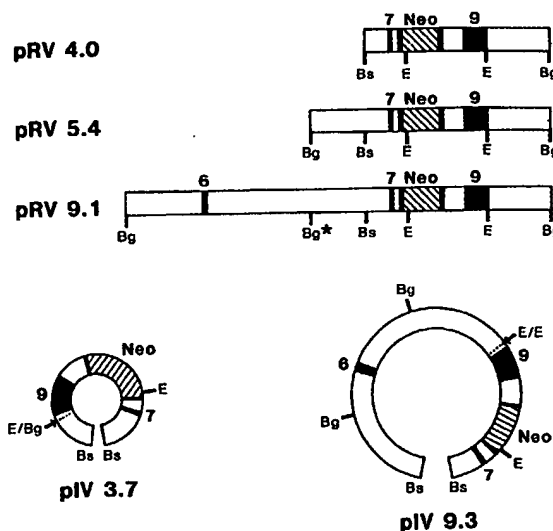


Figure 4. Targeting Vectors

The vectors were constructed as described in Experimental Procedures. The closed boxes represent *Hprt* exons, numbered according to the map of Melton et al. (1984). Open boxes represent introns and 3'-noncoding sequences. The crosshatched box represents the *neo<sup>r</sup>* gene from pMC1Neo inserted onto the eighth exon of *Hprt*. The ends of each sequence on the diagram correspond to the site of insertion of each sequence into a pUC9-derivatized plasmid. Digestion of the plasmids with the appropriate restriction endonuclease released the *Hprt* sequences from the plasmid, creating the targeting vectors depicted above. The length of the *Hprt* sequences in each vector is as follows: pRV4.0, 4 kb; pRV5.4, 5.4 kb; pRV9.1, 9.1 kb; pIV3.7, 3.7 kb; pIV9.3, 9.3 kb. All vectors contain the poly(A) addition sequences from the *Hprt* gene. RV, replacement vector; IV, insertion vector; Bg, BglII; Bs, BstEII; E, EcoRI; the dotted line in each pIV designates the point of discontinuity of the *Hprt* gene due to the joining of the 5' and 3' ends during vector construction. Bg\*, the internal BglII site in pRV9.1, was eliminated by cleavage with BglII and filling in with Klenow fragment and dNTPs. This permits the excision of the replacement vector using the terminal BglII sites.

tions: nonselective media, to assess the total number of cells surviving electroporation; G418 media, to assay the fraction of survivors transformed by the *neo<sup>r</sup>*-containing vectors; and G418, 6-TG media, to select for cells simultaneously containing the *neo<sup>r</sup>* gene but lacking a func-



Table 2. Gene Targeting Using Sequence Replacement Vectors

Vector	Exp.	No. of Cells Surviving Electroporation	No. of G418 <sup>r</sup> Colonies	No. of G418 <sup>r</sup> + 6-TG <sup>r</sup> Colonies	G418 <sup>r</sup> + 6-TG <sup>r</sup>
					G418 <sup>r</sup>
pRV4.0	1	5.3 × 10 <sup>7</sup>	8.1 × 10 <sup>4</sup>	2	1/40,000
	2	4.3 × 10 <sup>7</sup>	4.3 × 10 <sup>4</sup>	2	1/21,500
pRV5.4	1	11.0 × 10 <sup>7</sup>	6.9 × 10 <sup>4</sup>	10	1/6,900
pRV9.1	1	7.8 × 10 <sup>7</sup>	3.0 × 10 <sup>4</sup>	32	1/950

ES cells were transfected with 25 µg/ml of linearized pRV4.0, pRV5.4, or pRV9.1. The conditions for electroporation, cell culture, selection for G418<sup>r</sup> cell lines, and selection for G418<sup>r</sup>, 6-TG<sup>r</sup> cell lines are described in Experimental Procedures.

tional *Hprt* gene. As discussed below, in cells showing the G418<sup>r</sup>, 6-TG<sup>r</sup> phenotype, the endogenous *Hprt* gene was inactivated by the targeted replacement of the endogenous sequence with the *neo<sup>r</sup>*-recombinant sequence.

In Table 2, we summarize the ability of the three different sequence replacement vectors to confer G418<sup>r</sup> and G418<sup>r</sup>, 6-TG<sup>r</sup> upon ES cells. Although the three vectors transform ES cells to G418<sup>r</sup> at a similar frequency (~1/1000), there is a marked difference in their ability to generate G418<sup>r</sup>, 6-TG<sup>r</sup> colonies. Of the G418<sup>r</sup> cells transformed with the smallest vector, pRV4.0, only 1/40,000 to 1/20,000 showed resistance to 6-TG. However, in those cells transformed to G418<sup>r</sup> by the largest vector, pRV9.1, 1/950 also showed the 6-TG<sup>r</sup> phenotype. Transformation by the intermediate-sized vector, pRV5.4, gave an intermediate frequency of 6-TG resistance, with 1/7,000 G418<sup>r</sup> colonies showing the 6-TG<sup>r</sup> phenotype.

To show that the G418<sup>r</sup>, 6-TG<sup>r</sup> phenotypes were the result of gene-targeting events, the *Hprt* genes from 23 independently isolated G418<sup>r</sup>, 6-TG<sup>r</sup> cell lines were characterized by Southern transfer analysis. In every instance (23/23) the cells were shown to contain a single copy of the *Hprt* gene harboring the *neo<sup>r</sup>* gene in exon 8. This result was seen in cells transformed with either pRV4.0, pRV5.4, or pRV9.1. Also, as expected, *Hprt* enzymatic activity could not be detected in these cell lines. As judged by their ability to incorporate [<sup>3</sup>H]hypoxanthine into their nucleic acid, these cells contained 10<sup>3</sup>- to 10<sup>4</sup>-fold less activity than the parental ES cell line (data not shown).

An example of the Southern transfer analysis is shown in Figure 5A, in which the G418<sup>r</sup>, 6-TG<sup>r</sup> cell line EP17-2M is compared with the parental ES cell line. DNA from each line was digested with the enzymes, BglII, EcoRI, or BglII plus EcoRI, electrophoresed in agarose, and transferred to nitrocellulose paper. The paper was then hybridized with radiolabeled DNA containing 1 kb of *Hprt* sequence (Figure 5A, probe A).

As predicted from the restriction map of the cloned *Hprt* gene (see Figure 5A), digestion of the ES DNA with BglII, EcoRI, or BglII plus EcoRI isolates sequences homologous to the *Hprt* probe on fragments of lengths 5.4 kb, 9.3 kb, or 3.7 kb, respectively. The digestion pattern of the DNA from the G418<sup>r</sup>, 6-TG<sup>r</sup> cell line is quite different, showing fragments of 6.4 kb, 8.3 kb, and 2.7 kb. As illustrated in Figure 5C, this pattern would exist if the endogenous *Hprt* gene had been replaced by vector sequences containing the *neo<sup>r</sup>* gene insertion. Because the *neo<sup>r</sup>*

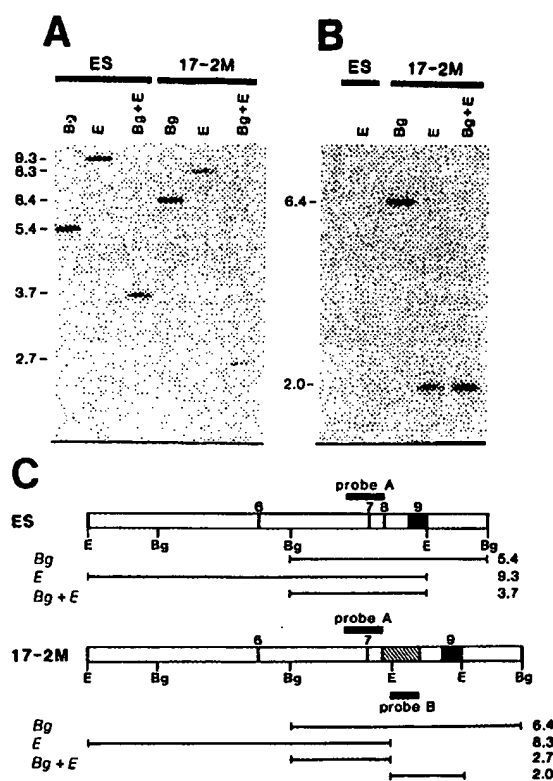


Figure 5. Southern Transfer Demonstration of Sequence Replacement DNA was purified from each cell line and digested with restriction endonuclease. DNA (7 µg) was loaded onto an agarose gel, electrophoresed, transferred to nitrocellulose, and hybridized to <sup>32</sup>P-labeled DNA probes. ES refers to DNA from the parental, wild-type ES cell line. 17-2M refers to DNA from a G418<sup>r</sup>, 6-TG<sup>r</sup> cell line transformed with the replacement vector, pRV4.0. (A) Probe A was a 1 kb fragment of mouse *Hprt* DNA extending from the BstEII site in intron 6 to the Scal site in exon 8. (B) Probe B was the 1200 bp *Ddel* fragment of the *neo<sup>r</sup>* structural gene from the plasmid, pRH140 (Thomas and Capecchi, 1986). The lengths of the fragments are given in kb and were determined by the coelectrophoresis of λ and plasmid fragments of known lengths. (C) A schematic representation of the Southern transfer data. The top map represents the 3' end of the *Hprt* gene from ES cells; the bottom represents the *Hprt* gene from the 17-2M cell line. Open boxes represent introns, closed boxes represent exons, and the crosshatched box represents the *neo<sup>r</sup>* gene. Beneath each gene is shown the restriction fragments hybridizing to the probes. Bg, BglII; E, EcoRI.

gene insert has no BglII site, the size of the BglII fragment is increased by the size of the insert (1 kb). However, because the *neo<sup>r</sup>* gene does contain an EcoRI site, its pres-

Table 3. Gene Targeting Using Sequence Insertion Vectors

Vector	Exp.	No. of Cells Surviving Electroporation	No. of G418 <sup>r</sup> Colonies	No. of G418 <sup>r</sup> + 6-TG <sup>r</sup> Colonies	G418 <sup>r</sup> + 6-TG <sup>r</sup> G418 <sup>r</sup>
pIV3.7	1	$8.1 \times 10^7$	$5.7 \times 10^4$	3	1/19,000
pIV9.3	1	$0.74 \times 10^7$	$0.42 \times 10^4$	3	1/1,400
	2	$4.1 \times 10^7$	$2.25 \times 10^4$	21	1/1,100

ES cells were transfected by electroporation with 25  $\mu$ g/ml of linearized pIV3.7 or pIV9.3. The conditions for electroporation, cell culture, selection for G418<sup>r</sup> cells, and selection for G418<sup>r</sup>, 6-TG<sup>r</sup> cells are described in Experimental Procedures.

ence introduces a new EcoRI site into the *Hprt* gene, resulting in the production of a smaller EcoRI fragment.

The interpretation is further verified when the same DNAs are hybridized to sequences from the *neo*<sup>r</sup> gene (Figures 5B and 5C, probe B). As expected, the parental cell line contains no *neo*<sup>r</sup> homology. The G418<sup>r</sup>, 6-TG<sup>r</sup> derivative does show *neo*<sup>r</sup> homology at a site within the *Hprt* locus. Digestion of the DNA with BglII isolates the *neo*<sup>r</sup> gene on the same 6.4 kb fragment homologous to the *Hprt* probe. Because the *neo*<sup>r</sup> gene contains an EcoRI site at its 5' end, digestion with this enzyme separates the *neo*<sup>r</sup> gene from sequences homologous to the *Hprt* probe and thus creates a 2 kb fragment with *neo*<sup>r</sup> homology.

It should be noted that the G418<sup>r</sup>, 6-TG<sup>r</sup> cell line EP17-2M was isolated following transformation with pRV4.0. Although this vector lacks both the EcoRI and the BglII sites 5' to the *neo*<sup>r</sup> insertion site (see Figure 4), the cell line EP17-2M clearly has both sites at the predicted distance from the *neo*<sup>r</sup> gene. Such a positioning of two restriction sites is best explained by a targeted recombination event.

#### Gene Targeting with Sequence Insertion Vectors

The two sequence insertion vectors were linearized and introduced into ES cells by electroporation. These cells were then scored for total survivors, G418<sup>r</sup> survivors, and G418<sup>r</sup>, 6-TG<sup>r</sup> survivors. The results of these experiments are summarized in Table 3. The two vectors were equally competent in the ability to confer G418<sup>r</sup> resistance upon ES cells, but differed markedly in their ability to generate G418<sup>r</sup>, 6-TG<sup>r</sup> colonies. Whereas the smaller vector, pIV3.7, generates 6-TG<sup>r</sup> cells at a frequency of 1/20,000 G418<sup>r</sup> cells, the larger, pIV9.3, induces 6-TG<sup>r</sup> resistance at a frequency of 1/1,100 to 1/1,400 G418<sup>r</sup> cells. In all cases, the G418<sup>r</sup>, 6-TG<sup>r</sup> cells contained targeted mutations of their *Hprt* loci.

To show that gene targeting was responsible for generating the G418<sup>r</sup>, 6-TG<sup>r</sup> phenotype, we analyzed by Southern transfer analysis 12 cell lines transformed by pIV9.3. Unlike the case of the sequence replacement vectors in which all *Hprt* mutations were caused by the same type of event, inactivation of the *Hprt* gene by sequence insertion vectors occurred by two mechanisms. The majority of targeting events caused by pIV9.3 (9/12) were due to the insertion of the entire vector into the endogenous *Hprt* locus. The remaining targeting events were sequence replacements, resembling those events induced by the se-

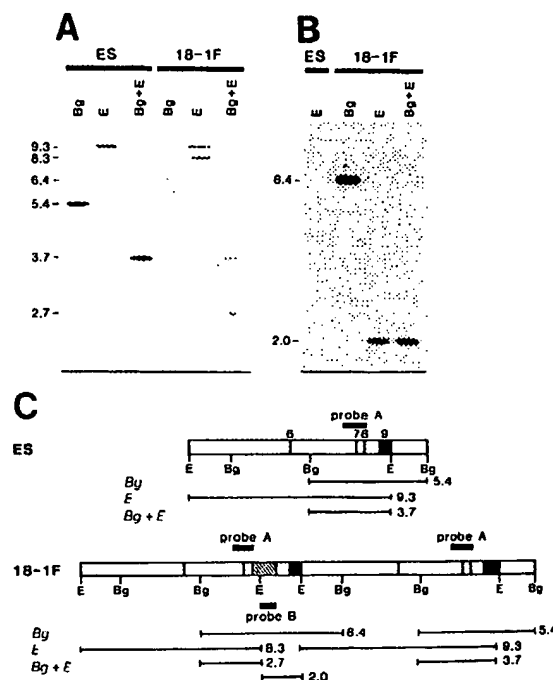


Figure 6. Southern Transfer Demonstration of Sequence Insertion Analysis was as described in Figure 5. ES is the parental cell line. 18-1F is a G418<sup>r</sup>, 6-TG<sup>r</sup> cell line transformed with the insertion vector pIV9.3. (A) Hybridization with probe A containing 1 kb of *Hprt* sequences from the BstEII site in intron 6 to the ScaI site in exon 8. (B) Hybridization with probe B, the *neo*<sup>r</sup> gene. (C) A schematic representation of the data. The top map represents *Hprt* sequences from the ES cell line. The bottom map represents sequences from the cell line 18-1F. The observed restriction fragments and their lengths are shown beneath each map. Bg, BglII; E, EcoRI.

quence replacement vectors. Examples of each event are shown in Figures 6 and 7.

In Figure 6 we show the Southern transfer pattern of cell line EP18-IF, a G418<sup>r</sup>, 6-TG<sup>r</sup> cell line transformed with pIV9.3. DNA from this cell line and DNA from the parental ES line were digested with BglII, EcoRI, or BglII plus EcoRI and probed with labeled *Hprt* sequences (Figure 6A, probe A). DNA from the parental cell line shows the 5.4, 9.3, and 3.7 kb fragments diagnostic of the wild-type *Hprt* gene. DNA from the G418<sup>r</sup>, 6-TG<sup>r</sup> cell line contains these same fragments, but also contains fragment of 6.4, 8.3, and 2.7 kb. These later fragments are characteristic of

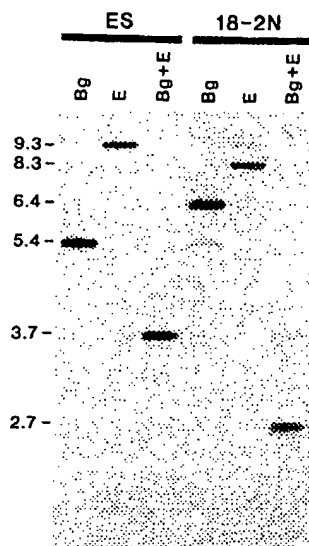


Figure 7. Southern Transfer Demonstration of Sequence Replacement Induced by a Sequence Insertion Vector

Analysis was as described in Figure 5. ES is the parental cell line; 18-2N is a G418<sup>r</sup>, 6-TG<sup>r</sup> cell line transformed with the insertion vector pIV9.3. The probe was the 1 kb *Hprt* fragment from the BstEII site in intron 6 to the ScaI site in exon 8 (probe A, Figure 5C). Bg, BglII; E, EcoRI. Note that the hybridization pattern is identical to that of cell line 17-2M (Figure 5A).

the *Hprt* gene containing the *neo'* gene in exon 8. One likely mechanism that would result in both fragments being recovered from the same cell is shown in Figure 6C. If the entire vector, pIV9.3 is inserted into the *Hprt* locus via homologous recombination, it will cause a 9.3 kb duplication of the *Hprt* sequences. The most 5' duplicated region will contain the *neo'* gene, whereas the most 3' duplicated region will contain wild-type sequences. Restriction enzyme digestions of this DNA will thus produce the hybrid configuration seen. This interpretation is further confirmed when the DNA from such a cell line is also probed with *neo'* sequences. As shown in Figure 6B, only 1 copy of the duplicated region contains *neo'* homology.

In 3/12 cell lines examined by Southern transfer analysis, this insertion pattern was not seen. Instead, the endogenous *Hprt* sequences appeared to have been replaced by the vector sequences containing the *neo'* insert. An example of one such cell line, EP18-2N, transformed by pIV9.3, is shown in Figure 7. DNA from this cell line and DNA from the parental ES line were digested with BglII, EcoRI, or BglII plus EcoRI and probed with *Hprt* sequences (Figure 7). The restriction pattern generated from this DNA is indistinguishable from that generated by digestion of DNA from cell line EP17-2M (see Figure 5A), a cell line transformed by a sequence replacement vector. Thus, sequence insertion vectors are also substrates for the sequence replacement reaction.

In examining the DNA products of the sequence replacement reactions, a low level of *Hprt* sequences lack-

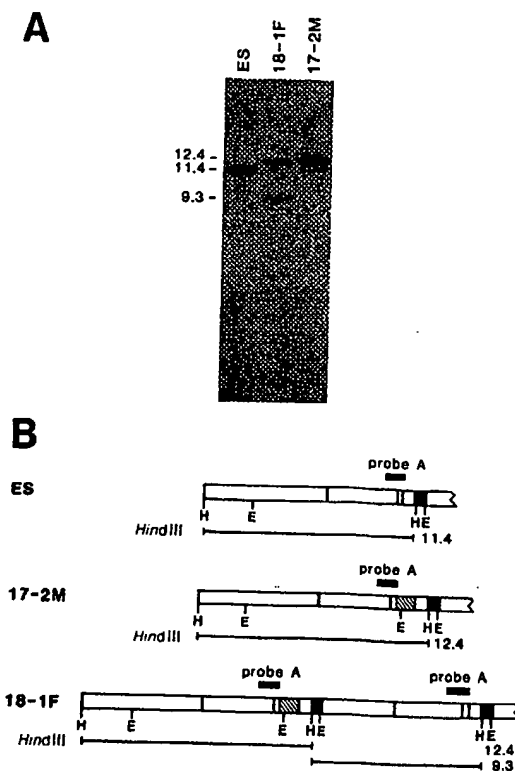


Figure 8. Southern Transfer Analysis of DNAs Digested with HindIII

DNAs were digested with HindIII and analyzed as described in Figure 5. ES is the parental cell line; 18-1F is a G418<sup>r</sup>, 6-TG<sup>r</sup> cell line transformed by sequence insertion; 17-2M is a G418<sup>r</sup>, 6-TG<sup>r</sup> cell line transformed by sequence replacement. (A) Cellular DNA was probed with *Hprt* probe A. (B) A diagram of the 3' region of the *Hprt* genes from the three cell lines analyzed. Bg, BglII; E, EcoRI; H, HindIII.

ing the *neo'* sequences is detected (see Figure 5A; Figure 7). These endogenous-length *Hprt* sequences come from the feeder cells upon which the ES cells are grown. The feeder cells are nonproliferating due to pretreatment with mitomycin C and represent a minor component of the total number of cells on the plate. However, the presence of such contaminating *Hprt* sequences presents a problem in our analysis of the sequence insertion events. In these latter events, the *neo'*-containing vector sequences lie adjacent to the endogenous sequences, and hybridization analysis revealed the presence of these two *Hprt* sequences in equal proportions (Figure 6A). We felt compelled to demonstrate that the endogenous-length *Hprt* copy detected in this Southern transfer was in fact adjacent to the inserted copy and not the result of feeder cell contamination.

To do this, DNA was digested with the restriction endonuclease HindIII and probed with *Hprt* sequence. Such a digestion permits a distinction to be made between a single copy endogenous sequence and a sequence adjacent to the inserted vector. As shown in Figure 8, the endogenous *Hprt* sequences, represented by the parental ES cell line, are contained on an 11.4 kb fragment. Se-

quences containing a single copy of the *Hprt* gene disrupted by the *neo'* gene, from cell line EP17-2M, are isolated on a 12.4 kb band. Digestion of DNA from the cell line EP18-1F, transformed by a sequence insertion vector, gives two *Hprt* fragments of equal intensity, one of 12.4 kb and one of 9.3 kb. This digestion pattern is quite consistent with a sequence insertion event.

## Discussion

We have analyzed 38 independent G418<sup>r</sup>, 6-TG<sup>r</sup> ES cell lines. Each of these cell lines was shown to have arisen from inactivation of the endogenous *Hprt* gene by homologous recombination with the introduced *neo'*-*Hprt* fragment. Spontaneous formation of G418<sup>r</sup>, 6-TG<sup>r</sup> cells was not detected (i.e., occurs at less than 1/10<sup>9</sup> cells per generation).

None of the G418<sup>r</sup>, 6-TG<sup>r</sup> cell lines contained extraneous copies of the *neo'*-*Hprt* recombinant vector integrated randomly within the ES genome. This greatly simplified the analysis of the targeting events in these cell lines and permitted an unambiguous interpretation of the results. The absence of extraneous copies of the input vector in the targeted cells should also simplify interpretation of additional studies that will entail establishing a correlation between the inactivation of specific genes with the resultant phenotypes.

Under our optimal conditions, we have observed a gene-targeting frequency, relative to the frequency of random integration of the input vector, of 1/1000. The parameters that we believe influenced the success of these experiments include using a *neo'* gene that is efficiently expressed in ES cells, maintaining the size of the *neo'* gene at a minimum, using extensive homology between the homing sequence and the target sequence, and removing, prior to transfection, unnecessary and nonhomologous sequences from the input vector.

This gene-targeting frequency is sufficiently high to be used for inactivating nonselectable genes. Direct screening, by Southern transfer analysis, for a gene-targeting event among 1000 candidate cell lines would not be exorbitant. Furthermore, gene-targeting enrichment procedures could be added to the protocol for using the *neo'* gene as a transfection tag. For example, a *neo'* gene lacking an enhancer or a poly(A) addition signal could be positioned within the homing sequence in such a way that homologous recombination with the target gene would juxtapose the defective *neo'* gene with the sequences required for effective expression. Random integration of the same vector into the recipient genome would not normally bring the required sequence sufficiently near the *neo'* gene to yield G418<sup>r</sup> colonies. Pilot experiments testing such procedures indicate that enrichment of several hundred fold for gene targeting compared with random integration should be attainable (unpublished results).

The gene-targeting frequency was observed to be very sensitive to the extent of homology between the exogenous and cognate endogenous sequence. A 2-fold increase in homology increased the gene-targeting frequency by 20-fold. Further increases in the extent of

homology may increase the gene-targeting frequency even more.

We have compared two classes of *neo'*-*Hprt* recombinant vectors, one that replaces endogenous sequences with exogenous sequences and another that inserts exogenous sequences into the endogenous sequence. Both classes exhibit comparable gene-targeting frequencies and are equally sensitive to the extent of homology with the endogenous target. We have termed the former sequence replacement vectors and the latter sequence insertion vectors. In 23/23 G418<sup>r</sup>, 6-TG<sup>r</sup> cell lines obtained by introducing the replacement vector, the endogenous *Hprt* gene was inactivated by sequence replacement. Of the 12 G418<sup>r</sup>, 6-TG<sup>r</sup> cell lines obtained with the insertion vector pIV9.3, 9 resulted from sequence insertion. In the remaining 3 the *Hprt* gene was inactivated by sequence replacement. The latter may result from a crossover occurring at points within the vector sequences rather than at both termini (Szostak et al., 1983). Though insertion vectors mediate mutagenesis via two pathways, they target predominantly by inserting into the endogenous gene.

The insertion vectors are technically more difficult to build. On the other hand, they may provide the means for generating a wider spectrum of mutant alleles. For example, by placing the *neo'* gene in the 3'-untranslated sequence, it can still be used as a transfection tag. In such a vector, the *neo'* gene could be linked to a wide spectrum of mutations, including point mutations, small insertions, or small deletions, in upstream exons. In the process of insertion of the *neo'* vector, these mutations would be concomitantly transferred into the endogenous gene.

When we initiated these experiments we had two concerns about using the *Hprt* gene as our target: it is expressed at a low level in ES cells, and it contains many repetitive DNA sequences. As in most cells, HPRT protein represents approximately 1/5,000 of the soluble protein (Hughes et al., 1975). Furthermore, repetitive DNA sequences are dispersed throughout both the *neo'*-*Hprt* recombinant vector and the *Hprt* gene. In fact, it is not a simple task to identify a suitable probe from the *Hprt* locus for Southern transfer analysis. The success obtained in targeting to the *Hprt* gene despite these handicaps may indicate that in the future we need not be so concerned with these parameters negatively influencing the gene-targeting frequency.

## Conclusion

We have demonstrated that we can inactivate by gene targeting a specific locus in the mouse genome. The protocol we have developed to inactivate the endogenous *Hprt* gene should be adaptable to other genes as well. We have also shown that ES cells are a suitable host for gene-targeting experiments. It is hoped that this combination of using ES cells as the recipient cell line and site-specific mutagenesis achieved by gene targeting will provide the means for generating mice of any desired genotype. An advantage of this scenario is that the first generation chimera will usually be heterozygous for the targeted mutation and that subsequent breeding can be used to gener-

ate the homozygous animal. Thus, only one of the two loci need be inactivated, and recessive lethals can be maintained as heterozygotes. If successful, this technology will be used in the future to dissect the developmental pathway of the mouse as well as to generate mouse models for human genetic diseases.

#### Experimental Procedures

##### Vector Construction

*Hprt* sequences were isolated from a  $\lambda$ , Charon 4A, library containing a partial *EcoRI*-digest of DNA from a mouse ARK cell line (the library was provided by Doug Foster, Ohio State University). The library was screened with a human cDNA *Hprt* probe (courtesy of C. Thomas Caskey). A recombinant phage containing the 9.3 *EcoRI* fragment encoding *Hprt* exons 6-9, as well as the 2.2 and 1.0 kb fragments 3' of the *Hprt* gene, was isolated. The 9.3 kb and 2.2 kb fragments were subcloned into pUC9 and converted by standard cloning methods into the targeting vectors. To introduce the *neo'* gene into *Hprt* exon 8, an 8 bp *XhoI* linker (New England Biolabs) was ligated into the *ScaI* site in exon 8.

The *neo'* vector pMC1Neo was created by the sequential ligation of its four functional domains (see Figure 2) into pUC9. The polyoma enhancer sequences were chemically synthesized on an Applied Biosystems model 380B DNA synthesizer and were flanked with *XhoI* (5' end) and *Sall* (3' end) restriction sites. To create the enhancer dimer used in pMC1Neo, the monomer units were ligated in vitro and the dimer was purified from a polyacrylamide gel. The HSV-*tk* promoter sequence from bases 92 to 121 was chemically synthesized and ligated in vitro to bases 122-218, isolated as an *EcoRI*-*PstI* fragment from the HSV-*tk* gene. The translational start sequence was synthesized chemically. The *neo'* gene was derived from the transposon Tn5. The structure of pMC1Neo was confirmed by DNA sequence analysis. pMC1Neo was designed such that the *neo'* gene and all its control elements could be removed as a 1 kb unit following digestion by *XhoI* and *Sall* and thus inserted into the *XhoI* site in the *Hprt* gene of the various targeting vectors.

Southern transfer analysis was performed as described previously (Thomas et al., 1988).

##### Isolation and Culturing of ES Cells

ES cells were isolated from C57B1/6 blastocysts as described by Evans and Kaufman (1981) except that primary embryonic fibroblasts (Doetschman et al., 1985) were used as feeders rather than STO cells. Briefly, 2.5 days postpregnancy mice were ovariectomized, and delayed blastocysts were recovered 4-8 days later. The blastocysts were cultured on mitomycin C-inactivated primary embryonic fibroblasts. After blastocyst attachment and the outgrowth of the trophectoderm, the ICM-derived clump was picked and dispersed by trypsin into clumps of 3-4 cells and put onto new feeders. All culturing was carried out in DMEM plus 20% FCS and  $10^{-4}$  M  $\beta$ -mercaptoethanol. The cultures were examined daily. After 6-7 days in culture, colonies that still resembled ES cells were picked, dispersed into single cells, and replated on feeders. Those cell lines that retained the morphology and growth characteristic of ES cells were tested for pluripotency in vitro. These cell lines were maintained on feeders and transferred every 2-3 days. For comparative purposes we have also used ES cell lines kindly provided by Martin Evans and Gail Martin. Cell lines from all three sources yielded targeted G418<sup>r</sup>, 6-TG<sup>r</sup> colonies at comparable frequencies. The G418<sup>r</sup>, 6-TG<sup>r</sup> cell lines are morphologically indistinguishable from the parental ES cells and retained their pluripotency in vitro (i.e., differentiate when grown on petri plates in the absence of a feeder layer and form embryoid bodies when grown in suspension).

**Electroporation and Isolation of G418<sup>r</sup> and G418<sup>r</sup>, 6-TG<sup>r</sup> Cell Lines**  
DNA was introduced into the ES cells by electroporation using the Promega Biotech X Cell 2000. Rapidly growing cells were trypsinized, washed in DMEM, counted, and resuspended in buffer containing 20 mM HEPES (pH 7.0), 0.37 mM NaCl, 5 mM KCl, 0.7 mM Na<sub>2</sub>HPO<sub>4</sub>, 6 mM dextrose, and 0.1 mM  $\beta$ -mercaptoethanol. Just prior to electroporation, the linearized recombinant vector was added. The cells were then exposed to a single, 625 V/cm pulse at room temperature, allowed to

remain in the buffer for 10 min, and plated onto feeder cells. For every experiment, aliquots of cells were removed before and after electroporation to measure colony-forming units; 40%-60% of the cells survived electroporation.

In a typical experiment  $10^7$  cells per vial were transfected by electroporation with 25  $\mu$ g/ml of linearized vector. Aliquots of cells were then subjected to one of three growth conditions: nonselective media, to evaluate the number of cells surviving electroporation; G418 media, to determine the fraction of survivors transformed by the *neo'* vector; and G418, 6-TG media, to select cells that had simultaneously acquired a *neo'* gene and lost a functional *Hprt* gene. For these experiments the ES cells were grown on mitomycin C-inactivated STO cells (obtained from Alan Bradley). To ensure inactivation, the STO cells were treated with 10  $\mu$ g/ml mitomycin C for 4 hr. Survival was less than  $1/10^9$  cells.

ES cells that were to be grown on nonselective medium were diluted  $4 \times 10^4$ -fold and  $8 \times 10^4$ -fold prior to plating onto 100 mm dishes containing the feeder cells. Cells that were to be subjected to growth on G418 medium or G418, 6-TG medium were diluted 200-fold and 28-fold, respectively, before plating onto feeders. To allow for expression of the *neo'* gene, the cells were first plated in nonselective medium and, 48 hr later, were transferred to G418-containing medium (250  $\mu$ g/ml). To allow for the decay of the endogenous HPRT activity, the cells that would eventually be subjected to G418, 6-TG selection were first plated in nonselective medium. Two days later they were transferred to G418 medium, and 5 days after electroporation they were transferred to G418, 6-TG (1  $\mu$ g/ml) medium.

At each transfer, the cells were trypsinized and placed on a new feeder plate in their respective medium. It is necessary to disperse the cells prior to subjecting them to selection because ES cells grow in tight clumps and cross-feed extensively. During this period of selection, the cells are dividing and it is necessary, for quantitative analysis, to keep track of the number of cell divisions. For this purpose, aliquots of cells from the same experiment were grown in nonselective medium, subjected to the same transfer protocol, and used to measure cell proliferation during this period. An outcome of the above protocol is that if a single targeting event occurs during the time of electroporation, one of the G418, 6-TG plates will yield a burst of 32-64 G418<sup>r</sup>, 6-TG<sup>r</sup> colonies; the rest of the plates will contain no colonies. This is scored as a single event. Each of the G418<sup>r</sup>, 6-TG<sup>r</sup> cell lines that we obtained came from such individual bursts, indicating that the targeting events occurred at the time of electroporation. The above scoring procedure underestimates the gene-targeting frequency since a burst of G418<sup>r</sup>, 6-TG<sup>r</sup> colonies on a given plate may have arisen from more than one event. For example, if the Poisson function was used to correct the pRV9.1 data shown in Table 2 for the expected number of plates that resulted from two events, then the gene-targeting frequency would be 1/800 G418<sup>r</sup> colonies rather than 1/950 G418<sup>r</sup> colonies.

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immune function, it is possible that 21-OH exerts an immunomodulatory role by regulating glucocorticoid biosynthesis. There may also be non-immunological effects; inbred mice display mating preferences depending on H-2 type, and can discriminate urine odours from H-2 congenic mice in Y-maze experiments<sup>18</sup>. H-2-associated differences in 21-OH activity would be expected to influence urinary levels of metabolites of both glucocorticoids and androgens, which might be perceived by mice. Such an influence on mating preference might encourage an advantageous mixing of mouse populations. An analysis of the regulation of the two 21-OH genes in different inbred mouse strains may help to answer these questions.

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## Correction of feline arylsulphatase B deficiency (mucopolysaccharidosis VI) by bone marrow transplantation

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Feline and human mucopolysaccharidosis VI (MPS VI or Maroteaux-Lamy syndrome) are inherited autosomal recessive deficiencies of lysosomal enzyme arylsulphatase B<sup>1-6</sup>. Affected cats and children exhibit lesions caused by incompetent degradation, retinal atrophy and excessive urinary excretion of dermatan facial dysmorphism, corneal stromal opacities, leukocyte granulation, retinal atrophy and excessive urinary excretion of dermatan sulphate—and usually die before adulthood<sup>7-11</sup>. Most attempts to

treat humans affected with MPS VI or other mucopolysaccharidoses have been ineffective or logistically prohibitive<sup>12-21</sup>, but allogeneic bone marrow transplantation (BMT) offers promise for cure of certain inborn errors of metabolism<sup>22-24</sup>. Engraftment of normal donor marrow may endow the enzyme-deficient recipient with a continuous source of enzyme-competent blood cells and tissue macrophages to facilitate degradation of stored substrate and to prevent genesis of further malformations. To test this hypothesis, we performed allogeneic BMT in a 2-year-old male Siamese cat with advanced MPS VI. Here we describe BMT-induced correction of this hereditary enzyme deficiency.

The MPS VI-affected 2-year-old male Siamese cat used in this experiment was the offspring of two known MPS VI heterozygotes<sup>3</sup>. A healthy female sibling with normal levels of arylsulphatase B activity was selected as marrow donor after it was found to be histocompatible with the affected cat on the basis of one and two-way mixed leukocyte nonreactivity—the sole criterion available for determination of feline histocompatibility (for methods see Fig. 1). Total-body irradiation was delivered to the affected recipient cat as a single 7.0-Gy dose at a rate of 2.5 Gy min<sup>-1</sup> with a 6-MeV linear accelerator after selective decontamination of its intestinal tract with oral neomycin and polymyxin B for 7 days. At 24 h post-irradiation, female sibling donor bone marrow cells (2 × 10<sup>6</sup> per kg body weight) were injected into the jugular vein of the irradiated MPS VI-affected cat (see Fig. 1). For 40 days thereafter, the cat was housed in a laminar flow sterile isolator where it received sterile food and water and its temperature, hydration, and fluid intake and output were monitored daily. Parenteral fluids and antibiotics were given as indicated by a deficit in hydration status and rectal body temperature greater than 39.2°C, respectively. Blood leukocyte and platelet concentrations, volume of packed red cells, leukocyte arylsulphatase B activity, and urinary glycosaminoglycan (GAG) excretion were determined before and after BMT (for methods see Figs 1, 2). Cyclosporin (15 mg per kg orally) was administered daily from 19 to 104 days post-BMT to prevent graft-versus-host disease. Karyotypic analysis of cultured bone marrow cells was performed 183 days after BMT.

Irradiation-induced myelotoxicity was evident on day 1 and extended to day 12 after BMT (Fig. 1). Beginning 18 days after BMT, rapid reconstitution by donor-origin granulocytes, monocytes and thrombocytes occurred. An early indication of donor-origin engraftment was a marked decrease in Alder-Reilly body-bearing neutrophils, and appearance of Barr body-bearing (female) neutrophils. Lymphocytes remained below pretransplant levels during cyclosporin therapy (days 19 to 104) but returned to normal levels thereafter. Urinary dermatan sulphate excretion decreased 2.5-fold by day 1 and 14.6-fold by day 232 after BMT (Fig. 2). Leukocyte arylsulphatase B activity increased 30-fold by 232 days after BMT. Both urinary dermatan sulphate excretion and leukocyte arylsulphatase B activity have maintained values within the normal range. Karyotypic analysis on day 183 post-BMT revealed a stable chimaera of 72.5% donor-origin (female) and 27.5% recipient-origin (male) cells.

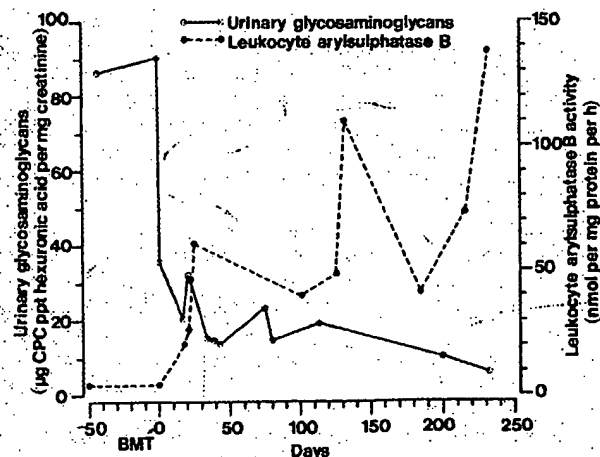
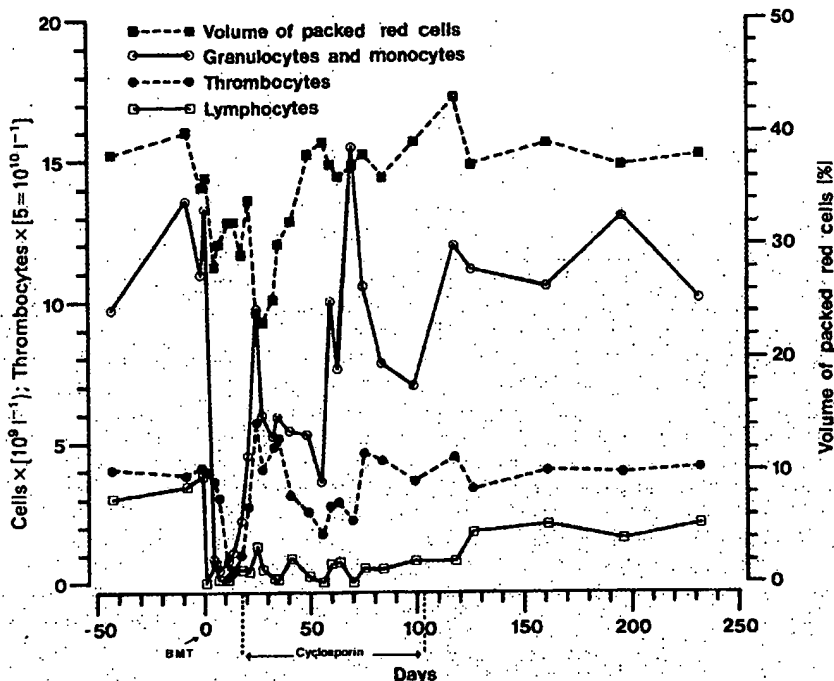
Clinical changes in the MPS VI-affected cat following restoration of arylsulphatase B activity by BMT have been complete resolution of corneal clouding and continuing resolution of facial dysmorphism (Fig. 3). Subjective changes in the cat after BMT include improved ability to walk and increased movement of the head, neck and mandible (for example, restored capacity to masticate dry food), increased suppleness of the haircoat and improved demeanour.

While the logic behind treating heritable disorders of bone marrow-derived cells with allogeneic BMT is evident (reviewed in ref. 23), the rationale for treating multisystemic lysosomal storage diseases, such as MPS VI, with allogeneic BMT is less clear. Implicit in the application of BMT therapy for conditions in which the biochemical defect is expressed in organs not derived from bone marrow, is the belief that either the adopted enzyme-replete cells will transfer enzyme to cells of the several involved organs<sup>25-28</sup>, or that bone marrow-derived cells are

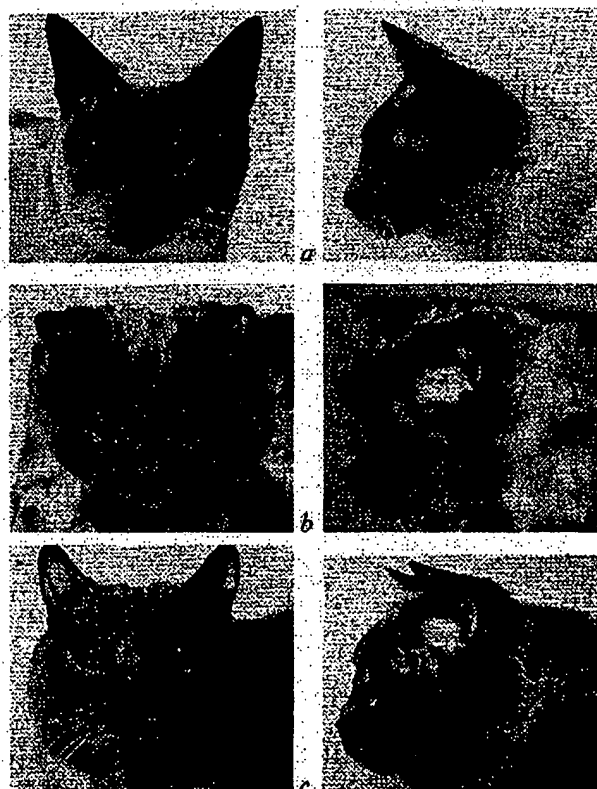
**Fig. 1** Peripheral blood cell numbers in the MPS VI-affected cat marrow transplanted with normal allogeneic feline bone marrow. Total-body irradiation-induced myelotoxicity (days 0-18) followed by bone marrow transplantation-mediated haematological reconstitution (days 18-25) and stabilization (days 25-250).

**Methods:** At the times indicated, complete blood counts and serum chemistry evaluations were performed. Ficoll-metrizoate ( $1.077 \text{ g cm}^{-3}$ )-separated leukocytes from each of four siblings of the MPS VI-affected cat were assayed for one and two-way mixed leukocyte reactivity against similarly separated lymphocytes of the affected cat according to a modification of Peck and Bach<sup>26</sup>. Lymphocytes ( $2 \times 10^5$ ) were mixed in 96-well plates either with  $2 \times 10^5$  mitomycin C-treated ( $2 \mu\text{g}$  mitomycin C per  $10^6$  lymphocytes, 45 min incubation at  $37^\circ\text{C}$ ) or with  $2 \times 10^5$  non-mitomycin C-treated leukocytes and incubated in a  $37^\circ\text{C}$ , humidified, 5%  $\text{CO}_2$  atmosphere for 7 days.

Eighteen hours before collection,  $0.5 \mu\text{Ci}$  of  $^3\text{H}$ -thymidine was added to each well. The cat with the lowest stimulation index was selected as the marrow donor. Marrow was obtained from the ketamine-anaesthetized donor by aspiration from both femurs and humeri. The cells were diluted in Hank's balanced salt solution, centrifuged at  $1,000g$  for 10 min, the surface fat layer was removed, and the buffy coat cells were separated by pipette aspiration and then filtered through six layers of sterile gauze. Viable mononuclear cells were enumerated with the aid of Trypan blue vital stain and  $2 \times 10^8$  mononuclear cells per kg infused into the jugular vein. Following haematological reconstitution (day 21) cyclosporin (a gift from J. F. Borel, Sandoz) was administered orally, 15 mg per kg daily. Serum trough levels of cyclosporin were measured at  $134 \mu\text{g l}^{-1}$  (University of Minnesota Hospitals, Outreach Program). Karyotypic analysis was performed as follows: aspirated bone marrow mononuclear cells from the affected cat were cultured for 4 days in  $25 \text{ cm}^2$  flasks as described<sup>37</sup>, medium containing nonadherent cells were transferred to a 15-ml conical centrifuge tube, incubated for 1 h at  $37^\circ\text{C}$  with  $4 \mu\text{g}$  of colchicine, and the pelleted cells were exposed to 5 ml of hypotonic ( $0.075 \text{ M}$ ) KCl solution for 10 min at  $37^\circ\text{C}$  and fixed with 1 ml of methanol:acetic acid (1:3). The final cell suspension was dropped on cleaned glass slides and dried at  $65^\circ\text{C}$ , and stained with a 2% Giemsa stain for 6-8 min. Forty metaphase chromosome were examined at  $\times 1,000$ .



**Fig. 2** Urinary excretion of glycosaminoglycans and leukocyte arylsulphatase B activity of the MPS VI-affected cat marrow transplanted with normal feline bone marrow. Urinary excretion of glycosaminoglycans decreased to the normal range (day +18 to day +232) as arylsulphatase B activity was installed in the mucopolysaccharidosis VI-affected cat by bone marrow transplantation. Urinary glycosaminoglycans were determined according to a modification of Pennock<sup>38</sup>. CPC ppt, cetylpyridinium chloride precipitate. The predominant glycosaminoglycan excreted pre-BMT was dermatan sulphate, as determined by thin-layer chromatography. Leukocyte arylsulphatase B activity was determined according to a modification of Baum *et al.*<sup>39</sup>.



**Fig. 3** a, Normal Siamese cat; b, arylsulphatase B-deficient sibling of the cat shown in a; c, the same animal 105 days after bone marrow transplantation. Complete resolution of corneal clouding and partial resolution of facial disfigurement can be seen.



responsible for degradation of the majority of systemic substrate under normal conditions. Support for one or both of these mechanisms is provided by the demonstrated correction of the galactosylceramidase deficit in the nerves of 'twitcher mice' when these nerves are grafted into normal mice<sup>29</sup>.

Although allogeneic BMT has been performed in children with various lysosomal storage diseases, the long-term effectiveness of this treatment is unknown<sup>30-35</sup>. Our results show significant and sustained improvement in the health of an MPS VI-affected cat after successful allogeneic BMT, which suggests that allogeneic BMT may provide curative therapy for human MPS VI. However, in those lysosomal storage diseases where significant neurological damage occurs, this method must be further evaluated before it can be conscientiously recommended.

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Investigations using this animal model of human disease could clarify the pathogenesis of MPS-induced lesions and would allow detailed analysis of the ability of BMT to prevent and correct pathological changes caused by arylsulphatase B deficiency and perhaps other inborn enzyme deficiencies.

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## Unusual abundance of vertebrate 3-phosphate dehydrogenase pseudogenes

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Only one gene coding for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), a key enzyme in the control of glycolysis, is known to be functional in man, mouse, rat and chicken<sup>1-6</sup>. The gene has been localized to chromosome 12 in human<sup>2,3</sup> and chromosome 6 in mouse<sup>4</sup>. Only a single mRNA species has been found in chicken<sup>7-9</sup> and rat<sup>10</sup>. However, analysis of genomic DNA blots of various species with a cloned GAPDH cDNA probe has revealed large differences in the level of reiteration, ranging from one to over 200 copies. On this basis, we have grouped these organisms into three classes according to the number of GAPDH-related sequences they contain; one class with a unique representation (chicken), another class of relatively low reiteration (10-30 copies in man, hare, guinea-pig and hamster) and a third class of high reiteration (>200 copies in mouse and rat). The third class represents the first reported occurrence of such an extreme number of pseudogenes related to an enzyme-coding gene and suggests that a dramatic amplification event took place between 15 and 25 million years ago.

As a preliminary to the screening of genomic libraries to obtain the gene, we probed Southern blots<sup>11</sup> of chicken and rat genomic DNA, respectively, with GAPDH cDNA clones from rat (pR GAPDH1)<sup>10</sup> and chicken (pGPD1)<sup>7</sup>. In chicken DNA, we obtain a simple pattern of *EcoRI* (10 and 15 kilobases (kb)) and *HindIII* (8, 1.7 and 0.5 kb fragments) (Fig. 1). The *HindIII* pattern is identical with that observed by Kuo *et al.*<sup>12</sup> and the *EcoRI* pattern is also in good agreement with previous reports<sup>6,12</sup>, despite the presence of a weaker additional band, probably reflecting some polymorphism at *EcoRI* sites. All these data support the existence of a single GAPDH sequence in the chicken genome. In contrast, however, Fig. 1 shows a large number of fragments homologous to GAPDH cDNA in the rat genome. This clone consists exclusively of coding sequences (spanning amino acids 261-324)<sup>10</sup>, so this hybridization pattern does not result from repetitive sequences in untranslated regions<sup>13</sup>. Moreover, the probe is so small as to make it extremely unlikely that the multiple bands would correspond to genomic fragments harbouring different exons. To eliminate the possibility that the pattern of multiple bands detected results from incomplete digestion of the genomic DNA, the same blots were rehybridized to a rat serum albumin cDNA clone, yielding a pattern similar to the one published for this gene<sup>14</sup> (data not shown). The stringency of washing conditions (0.2 × SSC, 60 °C) suggests that these multiple genomic bands are highly homologous to GAPDH. Indeed, the same pattern was observed when filters were washed either at 25 °C in 2 × SSC or at 73 °C in 0.2 × SSC (not shown). This is therefore a clear indication for a highly multigenic family of related sequences in the rat genome. To rule out a preferential reiteration of only part of the GAPDH sequence as observed previously in the human myoglobin gene<sup>15</sup>, we rehybridized mouse and rat Southern blots with a *PstI*-*HinFI* fragment excised from a full-length rat cDNA clone. This probe is 229 nucleotides long, including 74 5' untranslated and 155 coding nucleotides; it gave rise to the same complex pattern of bands (not shown), indicating that these

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haematopoietic microenvironment and haematopoiesis in four experiments was 1%, 4%, 5% and 2%, respectively. In two experiments in which only single CD34<sup>+</sup>, HLA-DR<sup>-</sup>, CD38<sup>-</sup> cells with relatively large forward light scatter were sorted no cell growth was observed, whereas in two other experiments single CD34<sup>+</sup>, HLA-DR<sup>-</sup>, CD38<sup>-</sup> cells with low forward light scatter, 5% and 12%, respectively, of the single cells gave rise to both a haematopoietic microenvironment and haematopoiesis. No cells were found that gave rise to only haematopoietic colonies or only a haematopoietic microenvironment.

Self renewal ability of the formed structures was studied by disrupting and removing the structures and the supernatant, and replenishing of the media with serum, IGF-1 and b-FGF. After 2 to 5 days of culture only degenerated cells and debris could be found; however, the identical differentiation process developed as for the originally sorted single cells. After 15 to 17 days of culture, similar structures appeared including the presence of haematopoietic cell colonies. The disruption of the microstructure could be repeated and both the structure and the colonies reappeared in an identical timely fashion. These results provide direct evidence for the existence of a single class of common stem cells which can sequentially differentiate into both the microenvironment and the haematopoietic stem cells of human bone marrow. The observation that the structures repeatedly reconstructed themselves after disruption and supplementation with the media, IGF-1 and b-FGF, strongly suggests that the common stem cell of the haematopoietic cells and the haematopoietic microenvironment possesses the ability to self-renew. The data indicate that in early development of

the haematopoietic system, common stem cells first generate stromal stem cells which give rise to a haematopoietic microenvironment which then induce the common stem cells to differentiate into the haematopoietic stem cells.

The identification of common stem cells of both the haematopoietic microenvironment and the haematopoietic stem cells offers exciting new possibilities to study the haematopoietic system. For example, purified common stem cells might be the ideal target for gene therapy. Moreover, the involvement of these unique cells in malignancies of the haematopoietic system and its microenvironment can now be explored. □

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## Reversal of pathology in murine mucopolysaccharidosis type VII by somatic cell gene transfer

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AN inherited deficiency of  $\beta$ -glucuronidase in humans<sup>1</sup>, mice<sup>2</sup> and dogs<sup>3</sup> causes mucopolysaccharidosis VII (Sly syndrome), a progressive degenerative disease that reduces lifespan (to an average of 5 months in mice<sup>2</sup>) and results from lysosomal storage of undegraded glycosaminoglycans in the spleen, liver, kidney, cornea, brain and skeletal system<sup>1-4</sup>. Bone marrow transplantation in mutant mice provides a source of normal enzyme ('cross-correction'<sup>5</sup>), which substantially improves the clinical condition and extends the average lifespan to 18 months<sup>6</sup>. Gene therapy by transfer of a  $\beta$ -glucuronidase gene into mutant haematopoietic stem cells is an alternative approach<sup>7,8</sup>, but it is not known whether the low expression of vector-transferred genes *in vivo*<sup>9,10</sup> would be sufficiently effective. Here we show that retroviral vector-mediated transfer of the gene to mutant stem cells results in long-term expression of low levels of  $\beta$ -glucuronidase which partially corrects the disease by reducing lysosomal storage in liver and spleen.

Bone marrow cells from mucopolysaccharidosis (MPS) VII mice were infected with the NTK-BGEO vector<sup>7,8,11,12</sup> (Fig. 1a) and transplanted intravenously into eight lethally irradiated adult MPS VII recipients. Spleens from two mice examined 11 days after transplantation contained  $\beta$ -glucuronidase-positive

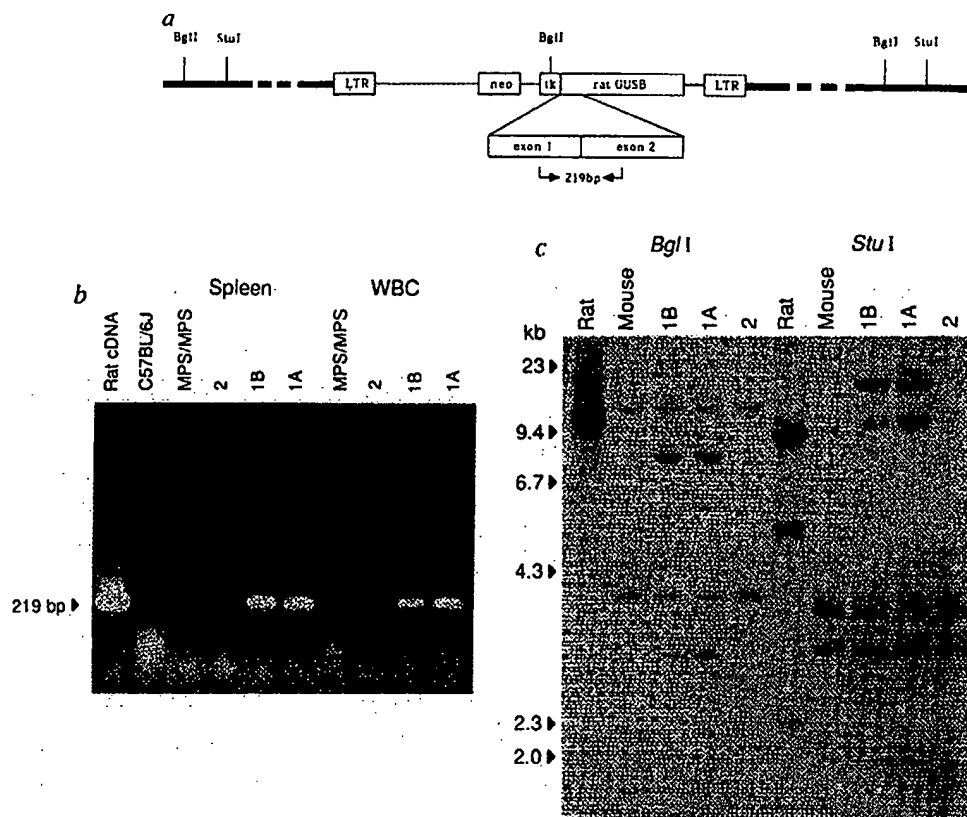
cells<sup>6,7,12,13</sup>. One of four animals alive 42 days after transplantation had detectable  $\beta$ -glucuronidase activity in circulating white blood cells. To demonstrate that long-term repopulating stem cells in this mouse contained vector proviruses, we pooled spleen and bone marrow cells and transplanted them into irradiated adult MPS VII mice. The two secondary recipients (mice 1A and 1B) and one of the three remaining primary recipients (mouse 2) survived longer than six months after transplantation, which indicated that they had been reconstituted by stem cells, and were killed for further evaluation.

Vector proviral sequences were detected in all three animals by polymerase chain reaction (PCR) (Fig. 1b), indicating that vector-infected stem cells had reconstituted the haematopoietic system of each mouse<sup>8</sup>. Analysis of vector provirus integration patterns suggested that the repopulating cells were derived predominantly from a small number of transduced stem cells<sup>14</sup> (Fig. 1c). The secondary recipients (1A and 1B), which appeared to have the same viral integration sites, were probably reconstituted by cells derived from common stem cell precursors in the primary donor<sup>15</sup>. The strength of PCR and hybridization signals suggest that a substantial proportion of the repopulating cells contained vector provirus.

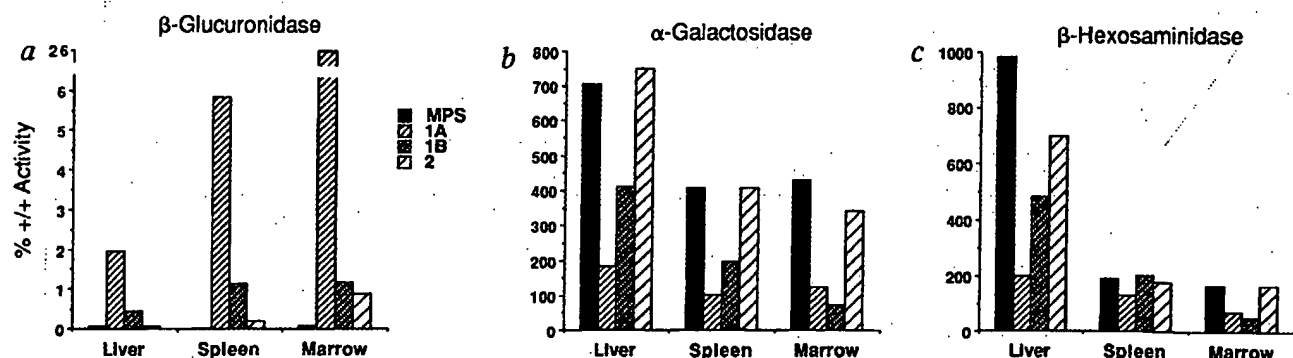
The level of  $\beta$ -glucuronidase expression was assayed in various organs, including liver, spleen and bone marrow (Fig. 2a). Mouse 1A had 6% of normal activity in spleen and lymph nodes, 2% in thymus and liver, 26% in bone marrow, and 1% or less in lung and kidney. Mouse 1B had 1-2% of normal activity in bone marrow and spleen, 9% in thymus, and less than 1% in lung and liver. In mouse 2,  $\beta$ -glucuronidase activity was only found in bone marrow and spleen and was less than 1% of normal.  $\alpha$ -Galactosidase and  $\beta$ -hexosaminidase activities were also measured as they are increased in untreated MPS VII mice<sup>2</sup> and correction of the rise after treatment by bone marrow transplantation is correlated with reduction of storage<sup>6</sup>. Mice 1A and 1B had reduced levels of  $\alpha$ -galactosidase in liver, spleen, and marrow (Fig. 2b), and of  $\beta$ -hexosaminidase in liver and marrow (Fig. 2c). No changes were apparent in mouse 2 (Fig. 2b and c).

# LETTERS TO NATURE

**FIG. 1** Vector infection, cell transplantation, and detection of vector proviral sequences in treated mice. **a**, Map of the  $\beta$ -glucuronidase (GUSB)-expression vector<sup>7</sup> showing restriction enzyme sites and location of oligonucleotide primers used for the PCR. The vector virus, which expresses normal levels of GUSB and restores substrate degradation to normal *in vitro*<sup>7,11</sup>, was produced in GP+E86 packaging cells<sup>19</sup> at  $\sim 10^6$  PFU per ml (G418 resistance). Stem cells were enriched from MPS VII bone marrow by density gradient centrifugation<sup>8,20</sup>, cultured in  $200 \text{ U ml}^{-1}$  each of recombinant mouse interleukin-3 and recombinant human interleukin-6 (GIBCO/BRL) for 96 h at  $37^\circ\text{C}$  and  $5\% \text{ CO}_2$  (refs 8, 21). During the final 48 h, cells were co-cultured with the packaging cells in  $8 \mu\text{g ml}^{-1}$  polybrene<sup>8,21</sup>.  $4 \times 10^5$  cells were injected intravenously into 8 irradiated (9 Gy) MPS VII recipients (32–52 days old)<sup>6,8</sup>. Two mice were analysed for GUSB expression at 11 days post-transplant. Two mice that died between 11 and 42 days and 2 that died between 42 and 180 days post-transplant were not analysed. At 42 days post-transplant, bone marrow and spleen cells were isolated from a mouse with GUSB-positive cells in blood and spleen, and  $1.7\%$  normal activity in liver.  $1.4 \times 10^7$  pooled spleen and bone marrow cells from this animal were injected intravenously into 2 irradiated (9 Gy) secondary recipients (mice 1A and 1B) (56 days old). **b**, Detection by PCR of proviral sequences in the DNA of spleen and white blood cells (WBC) of treated mice (1A, 1B and 2) at 6 months post-transplantation (221, 221 and 288 days old, respectively). The first lane contains the PCR product amplified from a plasmid containing the rat GUSB cDNA<sup>22</sup>; the second, third and seventh lanes show the results of amplifying genomic DNA from normal and untreated MPS VII mice. The reaction was done using previously described methods and oligonucleotides<sup>8</sup>. **c**, Southern blot of spleen DNA hybridized with the rat GUSB cDNA probe (89% nucleic acid identity with mouse GUSB). *Bgl*I cuts the vector in the thymidine kinase promoter, so the bands represent the 3' end of the vector and flanking genomic sequences. Two bands (about 4 and 12 kb) of the mouse GUSB gene hybridized with the rat probe. Four unique provirus integration fragments were seen as bands of  $\sim 3.5$ , 5, 8 and 15 kb in animals 1A and 1B



(the 5-kb band can be seen in the original autoradiogram). *Stu*I does not cut within the vector, therefore the bands include both 5' and 3' flanking DNA and the hybridizing fragments were expected to be much larger than the 7 kb of the vector<sup>7</sup>. Two bands of genomic GUSB were seen at  $\sim 3$  and 4 kb. Unique bands of  $\sim 10$  kb were present in animals 1A and 1B. The bands from animals 1A and 1B that migrate at  $>20$  kb probably included multiple provirus-containing fragments that were not resolved on the gel. No unique fragments were identified in mouse 2, but proviral sequences were present by PCR (see **b**). As the PCR signal and GUSB activity were low in this animal, there were probably too few cells from unique clones to produce a detectable signal on the Southern blot. The DNA was prepared, digested, electrophoresed on a 0.8% agarose gel, blotted and probed as described<sup>2,8</sup>.



**FIG. 2** Lysosomal enzyme activity in tissues of three treated mice and an untreated MPS VII control expressed as a percentage of the activity in

normal mice. The specific activity (nmol substrate cleaved per hour per mg protein) was determined on duplicate samples as described<sup>2,6</sup>.

Localization of  $\beta$ -glucuronidase activity in tissues<sup>6,13</sup> (Fig. 3) showed positive cells distributed throughout the liver and spleen in mice 1A and 1B (Fig. 3c, d, h, i). In liver, the activity was seen in sinusoidal lining cells (presumably Kupffer cells) and was not visible in hepatocytes. In mouse 2, a few small clusters of positive cells were seen in liver (Fig. 3e) but most of the tissue was negative. These results suggest that only a subset of

the donor cells were expressing the transferred gene, consistent with downregulation of vector expression<sup>9,10</sup> or the presence of uninfected donor cells.

The characteristic histopathological feature of MPS VII is the presence of large cytoplasmic vacuoles, representing lysosomes distended by accumulated undegraded glycosaminoglycans (GAGs)<sup>1-6,11,16</sup>. The reduction of lysosomal storage observed in

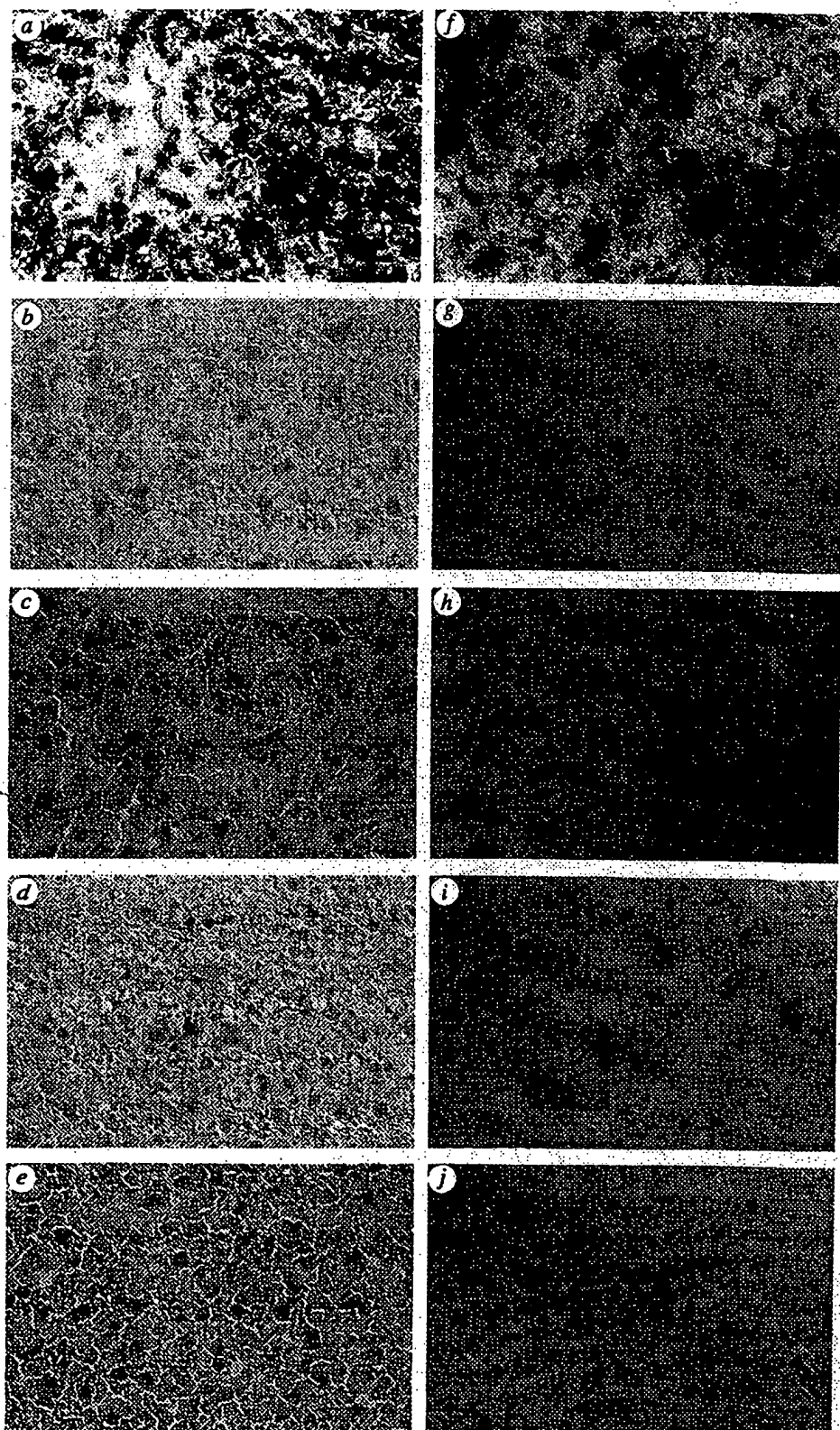


FIG. 3 Expression of GUSB enzymatic activity in tissues of MPS VII mice treated by gene transfer. The positive cells stain bright red<sup>6,7,12,13</sup>. a-e, Liver; f-j, spleen; a, f, normal; b, g, untreated MPS VII; c, h, mouse 1A; d, i, mouse 1B; e, j, mouse 2. Scale bar in a represents 20  $\mu$ m. The tissues to be tested for GUSB activity were isolated, frozen, cut into 10- $\mu$ m sections, assayed for activity using a histochemical reaction<sup>6,12,13</sup>, and photographed using differential interference microscopy<sup>13</sup>.



liver and spleen microscopically (Fig. 4) correlated with the levels of  $\beta$ -glucuronidase enzymatic activity (Figs 2 and 3). The liver and spleen of mouse 1A had 2% and 6% of the normal activity, respectively, and there was marked reduction in lysosomal storage (Fig. 4c, h). The hepatocytes were indistinguishable from normal by light microscopy (Fig. 4c) and electron microscopy (not shown). In mouse 1B the amount of storage

in liver and spleen cells was also reduced compared with untreated controls (Fig. 4d, i). In contrast, in mouse 2 only a few positive cells were seen in the *in situ* assay, very low or no activity was measurable by biochemical assay, and no reduction in storage was apparent histologically (Fig. 4e, j).

The dramatic decreases in lysosomal storage observed in liver and spleen demonstrate that low levels of  $\beta$ -glucuronidase in these tissues are sufficient to metabolize both the stored and newly produced GAGs. Hepatocytes had greatly reduced storage even though they appeared negative for  $\beta$ -glucuronidase activity histochemically (Fig. 3). Results were similar following normal bone marrow transplantation, when 15–18% of normal  $\beta$ -glucuronidase activity was present in liver but was detected in sinusoidal lining cells, not in hepatocytes<sup>6</sup>. Perhaps the *in situ* assay is not sufficiently sensitive to detect small amounts of cross-corrective enzyme in hepatocytes. Alternatively, proteoglycan turnover in the liver may be primarily a Kupffer cell function and MPS VII hepatocytes only store GAGs when Kupffer cells are metabolically blocked. Accordingly, vector correction of MPS VII Kupffer cells might restore the preferred pathway of proteoglycan turnover, and substrate stored in hepatocytes may decrease by nonspecific release of undegraded GAGs resulting from normal turnover of the endosome-lysosome compartment.

The therapeutic response is clearly dependent on the level of  $\beta$ -glucuronidase expression in the diseased mice. Here, gene transfer to haematopoietic stem cells resulted in the expression of low levels of  $\beta$ -glucuronidase and clearance of storage in liver and spleen, but not in kidney and cornea (data not shown). In contrast, the higher level of enzyme expressed after treatment by bone marrow transplantation reduces storage in all four tissues<sup>6</sup>. The pathology of the brain and skeleton is not corrected even by the higher levels of enzyme activity in recipients of normal marrow<sup>6</sup>, so different approaches<sup>13</sup> will be necessary to effect a complete cure. Nevertheless, the degree of correction achieved by low-level expression of the normal enzyme appeared to improve the clinical well-being of the animals in that mice 1A and 1B were more active and looked healthier than age-matched (8 months) untreated MPS VII mice. Mice that responded to gene therapy appeared similar to long-term recipients of bone marrow transplants in which lifespan is extended<sup>6</sup>.

The discovery that low levels of expression of the transferred  $\beta$ -glucuronidase gene can strikingly reduce storage suggests new approaches for treatment of lysosomal storage diseases. Partial replacement of bone marrow with a small number of vector-corrected stem cells may be sufficient to correct the metabolic defects in liver and spleen without inducing the severe complications associated

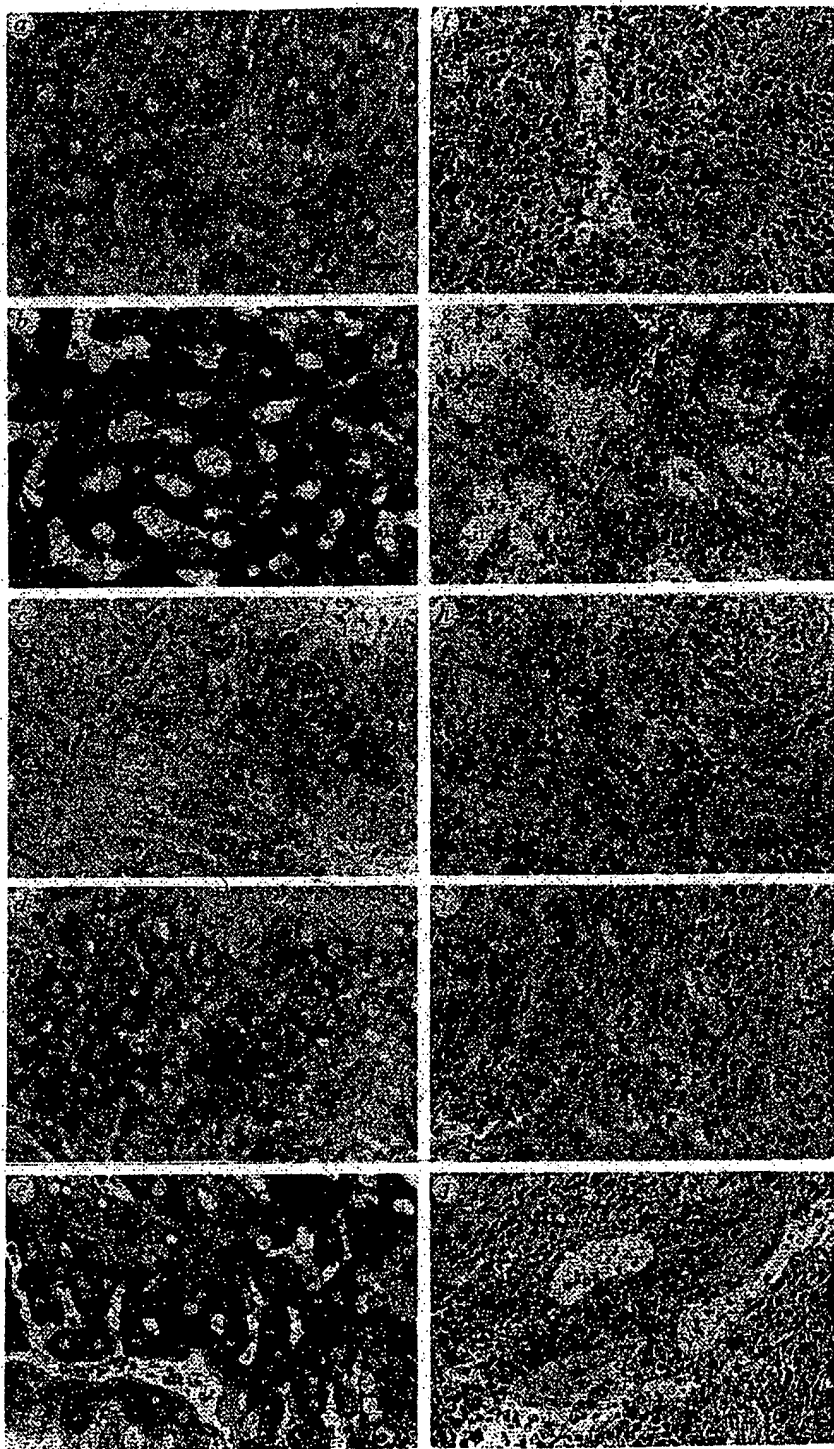


FIG. 4 Changes in lysosomal storage in liver and spleen of MPS VII mice after long-term haematopoietic reconstitution from vector-infected stem cells. Lysosomal storage is represented by the large cytoplasmic vacuoles. a–e, Liver; f–j, spleen; a, f, normal; b, g, untreated MPS VII; c, h, mouse 1A; d, i, mouse 1B; e, j, mouse 2. Scale bar in a represents 33  $\mu$ m. Tissues were fixed, embedded, sectioned (1  $\mu$ m), stained with toluidine blue and photographed as described<sup>2,8,16</sup>.

with bone marrow ablation. It may now be feasible to deliver therapeutically effective amounts of normal enzyme to patients by gene transfer to tissues such as muscle<sup>12</sup>, liver<sup>17</sup> or organoids<sup>18</sup>, in addition to haematopoietic stem cells. Our results indicate that the amount of functional enzyme that can be delivered, even though it is much lower than normal, may still be of therapeutic value to patients with mucopolysaccharidoses. □

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## Brain-derived neurotrophic factor rescues spinal motor neurons from axotomy-induced cell death

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**CURRENT** ideas about the dependence of neurons on target-derived growth factors were formulated on the basis of experiments involving neurons with projections to the periphery<sup>1,2</sup>. Nerve growth factor (NGF) and recently identified members of the NGF family of neuronal growth factors, known as neurotrophins, are thought to regulate survival of sympathetic and certain populations of sensory ganglion cells during development<sup>3-8</sup>. Far less is known about factors that regulate the survival of spinal and cranial motor neurons, which also project to peripheral targets. NGF has not been shown to influence motor neuron survival<sup>9,10</sup>, and whether the newly identified neurotrophins promote motor neuron survival is unknown. We show here that brain-derived neurotrophic factor (BDNF) is retrogradely transported by motor neurons in neonatal rats and that local application of BDNF to transected sciatic nerve prevents the massive death of motor neurons that normally follows axotomy in the neonatal period. These results show that BDNF has survival-promoting effects on motor neurons *in vivo* and suggest that BDNF may influence motor neuron survival during development.

We first examined the ability of spinal motor neurons to retrogradely transport BDNF in neonatal rats. Recombinant human BDNF was produced and purified as described<sup>11</sup> and iodinated<sup>12</sup>. <sup>125</sup>I-labelled BDNF was injected into the right lower

TABLE 1 Motor neuron counts in L4-5 spinal cord segments of 6-day-old rats after right sciatic nerve transection on postnatal day 1

Animal	Factor	No. of motor neurons		Survival (R/L × %)	Mean ± s.d.
		Right	Left		
1	BDNF	1,088	1,134	96	92 ± 6.6*
2	BDNF	922	1,098	84	
3	BDNF	1,065	1,216	88	
4	BDNF	1,117	1,142	98	
5	Cytochrome c	949	1,588	60	60 ± 5.7
6	Cytochrome c	855	1,298	66	
7	Cytochrome c	716	1,191	60	
8	Cytochrome c	531	1,022	52	

Motor neuron survival is presented as the percentage of counts on the right (R; lesioned side) over left (L; non-lesioned side).

\*Significantly more motor neurons survived after treatment with BDNF than after treatment with cytochrome c ( $P < 0.0002$ , Student *t*-test, two tails).

leg of 2-day-old rats and retrograde transport was examined by emulsion autoradiography of lumbar spinal cord sections (Fig. 1). Dense accumulation of silver grains was found over motor neurons in the ipsilateral spinal motor column (Fig. 1a). No silver grains above background level were observed in the contralateral spinal cord. Co-injection of a 50-fold excess of unlabelled BDNF completely blocked transport of <sup>125</sup>I-labelled BDNF (Fig. 1b). <sup>125</sup>I-labelled cytochrome c, which has physical chemical properties that are similar to BDNF, was not retrogradely transported by motor neurons after injection into the leg (data not shown). These results show that the ability of motor neurons to transport BDNF in a specific receptor-mediated fashion, previously demonstrated in adult rats<sup>11</sup>, is present by postnatal day 2.

The demonstration of specific retrograde transport of BDNF prompted us to ask whether this molecule has biological effects on spinal motor neurons. A rapid and reproducible motor neuron cell death occurs after sciatic nerve axotomy in neonatal rats<sup>13,14</sup>. Motor neurons with axons in the sciatic nerve account for 40% of motor neurons in the lower lumbar spinal cord and virtually all are lesioned by axotomy in the first 48 h of life<sup>15</sup>. We therefore used this experimental paradigm to assess the protective effects of BDNF on spinal motor neurons. BDNF (at 1 mg ml<sup>-1</sup> in PBS, corresponding to a dose of 5 µg g<sup>-1</sup> body weight) was subcutaneously injected into the right hindleg of newborn rats (day 0) within 2-3 h after birth. On day 1, the right sciatic nerve was cut near the obturator tendon in the thigh and a 3 × 3 × 3 mm<sup>3</sup> Gelfoam presoaked in BDNF (1 mg ml<sup>-1</sup> in PBS) was implanted next to the proximal sciatic nerve stump. In addition we injected 5 µl of the same BDNF solution into the lesion site daily during days 4-5 in order to supply additional BDNF to the transected motor axons. Control animals received cytochrome c in the same dosages administered in exactly the same fashion. Animals were killed on day 6. Motor neurons were counted on both sides of lumbar segments L4 and L5 as described previously<sup>10</sup>.

Results of this analysis are shown in Fig. 2 and Table 1. Figure 2 shows representative cross-sections of lumbar spinal cords from a control animal that received cytochrome c (Fig. 2a) and an animal that received BDNF (Fig. 2b). Spinal motor neurons on the non-lesioned side (left; open arrows) and on the side of the sciatic nerve section (right; solid arrows) are shown. The loss of motor neurons after sciatic nerve section in control animals treated with cytochrome c is apparent. In contrast, in BDNF-treated animals, most motor neurons survived axotomy over the 7-day period of the experiment. Cell counts from control and experimental animals showed that 40% of motor neurons were lost in cytochrome c-treated animals compared with only 8% loss in animals that received BDNF (Table 1). These data show that BDNF largely prevents the death of motor neurons

# Transplantation

## ARTICLES

### CORRECTION OF LYSOSOMAL ENZYME DEFICIENCY IN VARIOUS ORGANS OF $\beta$ -GLUCURONIDASE-DEFICIENT MICE BY ALLOGENEIC BONE MARROW TRANSPLANTATION<sup>1</sup>

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The correction of lysosomal enzyme deficiency was investigated for various organs of  $\beta$ -glucuronidase-deficient C3H/Rij mice after allogeneic bone marrow transplantation from an enzymatically normal donor strain (C57BL/Rij). In the hemopoietic organs, the enzyme level increased to levels found in donor mice. In lung, kidney, liver, and peripheral nervous tissue, a significant increase in enzyme activity was seen to levels intermediate between those of donor and recipient. Increased enzyme activity was maintained throughout the observation period of 150 days. In skeletal muscle tissue, enzyme levels tended to be higher in recipient mice, but this increase was not significant for all data points. Bone marrow transplantation failed to significantly affect enzyme activity in central nervous system tissue. These data suggest that beneficial effects expected from bone marrow transplantation for lysosomal enzyme deficiencies depend on the type of tissue involved in the disease. In diseases severely affecting the central nervous system, cure may not be expected from bone marrow transplantation alone, whereas in diseases with only minimal central nervous system involvement, alleviation or prevention of clinical symptoms may occur.

In lysosomal storage diseases, substrate accumulates in the lysosomes due to deficiency of a lysosomal enzyme (1). Enzyme replacement is the only rational means of therapy today. Administration of purified enzyme or plasma infusions (2-4) have resulted in limited success only: in most cases, the foreign protein is rapidly cleared from the circulation due to uptake by the reticuloendothelial system (5). In the past few years, attempts were made to treat patients with certain lysosomal storage diseases by bone marrow transplantation (BMT)\* (6, for recent reviews: 7-9). Effects of BMT in animal models are

reported, e.g., in  $\beta$ -glucuronidase-deficient mice (10), Twitcher mice (11), and the feline Maroteaux-Lamy model (12). After BMT, the blood cells and tissue macrophages are replaced by donor-derived cells. These enzymatically normal cells serve as a continuous source of enzyme. Immunological reactions against the enzyme have not been observed after BMT. An increase in enzyme activity after BMT has been reported in the plasma and blood cells of various patients (6, 13-16). In a Maroteaux-Lamy syndrome patient (13) and in the  $\beta$ -glucuronidase-deficient C3H mouse model (10), increased enzyme activity has also been reported in the liver.

In several storage diseases—e.g., mucopolysaccharidoses—hepatosplenomegaly (6, 13, 17) and corneal clouding (6, 17) disappeared after transplantation of normal bone marrow. Except in the feline Maroteaux-Lamy model (12), marked response of bone tissue has not been reported. In diseases with severe involvement of the central nervous system (CNS)—e.g., metachromatic leukodystrophy—a significant beneficial effect on the neurological symptoms has not been observed (18-19), although the follow-up period was too short to draw firm conclusions. Also, in the galactosyl-ceramidase-deficient Twitcher mouse, improvement of CNS symptoms is absent after BMT, both clinically and histologically (11). These data indicate that the clinical results of BMT for the treatment of lysosomal storage diseases depend on the type of tissue that is most severely affected.

In the present study, the organ distribution pattern of the donor enzyme after BMT is investigated by using the  $\beta$ -glucuronidase-deficient C3H/Rij mouse. Activities of two control lysosomal enzymes, hexosaminidase and  $\beta$ -D-galactosidase, was determined to study whether aspecific side effects of BMT influenced the lysosomal enzyme activities. Although C3H mice have very low levels of  $\beta$ -glucuronidase, clinical symptoms do not occur. These mice have a normal life span, and the presence of storage products has been reported only in livers of older C3H mice (20). Using the same mouse model (10), Slavin and Yatziv reported an increase in enzyme activity in plasma and liver after BMT.

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\* Abbreviations used: BMT, bone marrow transplantation; CNS,

central nervous system; HBSS, Hanks' balanced salt solution; MoAb, monoclonal antibody; PNS, peripheral nervous system.

## MATERIALS AND METHODS

**Mice.** Inbred, specific-pathogen-free, 8-12-week-old, female C3H/Rij (C3H) and C57BL/Rij (C57BL) mice were used, both as recipients of bone marrow grafts and as bone marrow donors. C3H mice have very low levels of  $\beta$ -glucuronidase (10) and C57BL mice are enzymatically normal (Table 1).

**Bone marrow transplantation.** Bone marrow was obtained by flushing femurs and tibias of donor mice with HEPES-buffered Hanks' balanced salt solution. One day after lethal total-body irradiation ( $^{137}\text{Cs}$  gamma rays, 9.5 Gy for C3H and 10.5 Gy for C57BL mice),  $10^7$  nucleated bone marrow cells were injected intravenously in the tail vein of recipient mice.

**Assay for chimerism.** Chimerism of nucleated bone marrow cells was assayed by using a monoclonal antibody (MoAb) that binds to granulocytes of C57BL origin, but not to those of C3H origin (Fig. 1A). Bone marrow cells from C3H and C57BL mice, and from chimeras were assayed.  $10^6$  Bone marrow cells were incubated with anti-GM 1.2 MoAb (NEI-301, New England, 50  $\mu\text{l}$  of antibody diluted 100 times in Hanks' balanced salt solution (HBSS) containing 5% fetal calf serum and 0.02% sodium azide) on ice for 45 min. The cells were washed with 0.5 ml HBSS and pelleted. Subsequently, the cells were incubated with goat-antimouse IgG fluorescein isothiocyanate (Nordic, Tilburg, The Netherlands; 50  $\mu\text{l}$  50 times diluted in HBSS) for 30 min on ice, which binds to the GM1.2 MoAb. After washing, the cells were resuspended in 0.5 ml of HBSS and analyzed on a light-activated cell sorter (FACS II, Becton and Dickinson, Sunnyvale, CA). An electronic window was set around a population of bone marrow cells, consisting virtually exclusively of granulocytes (21). Fluorescence of the cells in the window was measured. The bone marrow cells of the mice tested at 35, 50, 75, 100, and 150 days after allogeneic BMT bound the GM1.2 MoAb (Fig. 1B), proving that they were true hemopoietic chimeras.

**Hydrolase assay.**  $\beta$ -glucuronidase was assayed on freshly frozen organ specimens. Animals were perfused with saline ( $\pm 150$  ml) to remove contaminating blood. In addition to  $\beta$ -glucuronidase, hexosaminidase and  $\beta$ -D-galactosidase were measured at 50 days after BMT. Hydrolase activities were measured in a standard fluorimetric assay as described (20). Formation of the 4-methylumbelliferone product was measured in a Perkin Elmer fluorometer, using 367 nm light for excitation and measuring 442 nm light for emission. Protein was determined by the method of Lowry (23). Enzyme activity was determined in bone marrow cells, spleen, leukocytes, plasma, kidney, liver, lung, and central nervous tissue and expressed as specific activity (nmol/hr/mg protein). For determination of enzyme activity in skeletal muscle tissue, the femoral quadriceps muscle was used, and for enzyme activities in peripheral nervous tissue (PNS), the sciatic nerve was used. For each data point, 5-8 animals were used.

**Statistical analysis.** For statistical analysis, the enzyme levels in the various organs of the allogeneic recipients, syngeneic recipients, and untransplanted animals were compared by the Student's *t* test. Differences observed were considered significant at *P* values of  $<0.01$ .

TABLE 1.  $\beta$ -Glucuronidase levels (nmolhr $^{-1}$ mg protein $^{-1}$ ; mean  $\pm$  SD) in various organs of female, adult, C3H, C57BL, and allogeneic transplanted C3H mice, 150 days after transplantation<sup>a</sup>

	C3H	C57BL	C57BL $\rightarrow$ C3H
Bone marrow	52.7 $\pm$ 6.1	188.1 $\pm$ 24.5 <sup>b</sup>	156.9 $\pm$ 10.2 <sup>b</sup>
Spleen	50.7 $\pm$ 3.4	146.5 $\pm$ 10.8 <sup>b</sup>	133.8 $\pm$ 5.9 <sup>b</sup>
Liver	12.8 $\pm$ 1.6	104.5 $\pm$ 7.9 <sup>b</sup>	24.5 $\pm$ 1.5 <sup>b</sup>
Lung	26.4 $\pm$ 2.3	99.9 $\pm$ 23.4 <sup>b</sup>	65.7 $\pm$ 14.8 <sup>b</sup>
Kidney	7.9 $\pm$ 1.0	35.6 $\pm$ 4.2 <sup>b</sup>	21.5 $\pm$ 6.7 <sup>b</sup>
Skeletal muscle	0.3 $\pm$ 0.1	1.7 $\pm$ 0.4 <sup>b</sup>	0.6 $\pm$ 0.2 <sup>b</sup>
CNS	1.4 $\pm$ 0.2	8.3 $\pm$ 0.6 <sup>b</sup>	2.1 $\pm$ 0.5 <sup>b</sup>
PNS	1.6 $\pm$ 0.1	7.0 $\pm$ 1.4 <sup>b</sup>	4.8 $\pm$ 0.4 <sup>b</sup>
Leukocytes	11.6 $\pm$ 3.2	79.8 $\pm$ 6.9 <sup>b</sup>	77.5 $\pm$ 6.9 <sup>b</sup>
Plasma <sup>a</sup>	2.2 $\pm$ 0.6	9.4 $\pm$ 2.0 <sup>b</sup>	4.4 $\pm$ 0.4 <sup>b</sup>

<sup>a</sup> 5-8 animals per group.

<sup>b</sup> *P* < 0.01 as compared with untransplanted C3H.

## RESULTS

**Hydrolase activities in untransplanted animals.** As shown in Table 1, the C3H mice have low values for  $\beta$ -glucuronidase activity in the various organs examined as compared with C57BL mice. No significant differences in enzyme activity were observed between C57BL and C3H mice for two other hydrolases,  $\beta$ -D-galactosidase and hexosaminidase (Table 2).

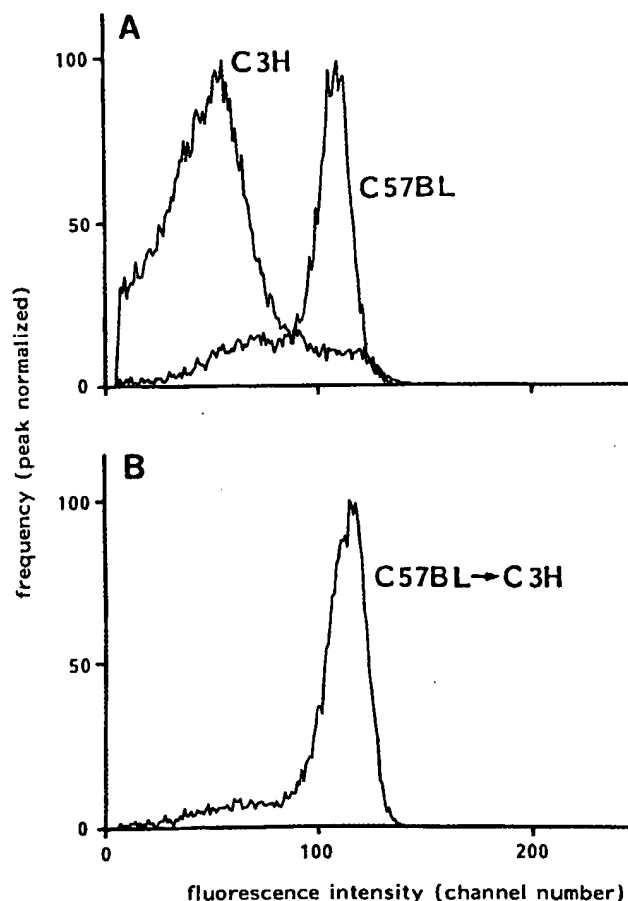


FIGURE 1. Frequency distribution of fluorescence intensities of anti-GM1.2 and anti-mouse IgG-FITC-labeled cells within the sorting window indicated in the Materials and Methods section. (A) cells of untransplanted C57BL and C3H mice. (B) cells of C57BL to C3H chimera.

TABLE 2. Levels (nmolhr $^{-1}$ mg protein $^{-1}$ ; mean  $\pm$  SD) of control enzymes in various organs of female, adult, C3H, and C57BL mice<sup>a</sup>

	$\beta$ -D-galactosidase		Hexosaminidase	
	C3H/Rij	C57BL/Rij	C3H/Rij	C57BL/Rij
Bone marrow	621.5 $\pm$ 104.3	346.3 $\pm$ 69.8	6014 $\pm$ 1083	4206 $\pm$ 638
Spleen	404.6 $\pm$ 5.5	408.2 $\pm$ 57.6	3048 $\pm$ 278	2959 $\pm$ 492
Liver	103.2 $\pm$ 8.6	132.3 $\pm$ 44.6	993 $\pm$ 154	948 $\pm$ 285
Lung	71.1 $\pm$ 12.4	146.8 $\pm$ 35.3	975 $\pm$ 304	1395 $\pm$ 373
Kidney	324.8 $\pm$ 14.7	304.6 $\pm$ 17.0	1695 $\pm$ 136	1382 $\pm$ 61
Skeletal muscle	2.0 $\pm$ 0.6	2.3 $\pm$ 0.2	62 $\pm$ 5	46 $\pm$ 5
CNS	58.1 $\pm$ 7.5	59.8 $\pm$ 9.8	1245 $\pm$ 93	1465 $\pm$ 87
PNS	34.2 $\pm$ 0.6	23.2 $\pm$ 2.6	804 $\pm$ 118	492 $\pm$ 42
Leukocytes	84.6 $\pm$ 57.6	107.5 $\pm$ 41.6	614 $\pm$ 60	879 $\pm$ 204
Plasma	28.9 $\pm$ 8.4	30.4 $\pm$ 6.9	3939 $\pm$ 542	3549 $\pm$ 622

<sup>a</sup> 5-8 animals per group.



**$\beta$ -glucuronidase activity in recipients of syngeneic grafts.** In general, significant differences in  $\beta$ -glucuronidase activity between untransplanted mice and recipients of syngeneic grafts did not occur (Figs. 2 and 3 [only data of bone marrow, liver, CNS, and PNS shown]). Only in liver tissue of C57BL mice that received a syngeneic graft, the enzyme activity significantly increased as compared with untransplanted animals (Fig. 2B). In  $\beta$ -D-galactosidase and hexosaminidase levels significant differences were not observed between the recipients of syngeneic grafts at 50 days after transplantation and the untransplanted controls (data not shown).

**$\beta$ -glucuronidase levels after allogeneic BMT.** After allogeneic BMT, increased  $\beta$ -glucuronidase levels occurred in various organs of C3H recipients. This increase was already significant ( $P < 0.01$ ) at day 35 after BMT and persisted during the observation period of 150 days after transplantation. In bone marrow (Fig. 2A), plasma, spleen, and peripheral blood leukocytes,  $\beta$ -glucuronidase activity increased to levels identical to those of donor mice (Table 1).

In the liver (Fig. 2B), lung, and kidney,  $\beta$ -glucuronidase activity increased significantly ( $P < 0.01$ ) to levels intermediate between those of C57BL and C3H (Table 1).

In skeletal muscle tissue, low specific  $\beta$ -glucuronidase activities were measured. There was a tendency toward higher levels in  $\beta$ -glucuronidase activity at all time points tested in allogeneic recipients as compared with syngeneic recipients as well as untreated controls. However, the differences were only statistically significant at day 75 after transplantation.

In the CNS, no significant increase in  $\beta$ -glucuronidase activity occurred after BMT (Fig. 3A); only a tendency toward higher enzyme levels as compared with syngeneic recipients and untransplanted mice at days 100 and 150 after BMT was seen. In this organ with low specific glucuronidase activity, contamination with plasma, even after perfusion, cannot be completely ruled out.

In the sciatic nerves, significantly higher  $\beta$ -glucuronidase activity was measured (Fig. 3B) in allogeneic transplanted mice as compared with untransplanted controls and syngeneic recipients.

Differences in activity of the two unrelated hydrolases, hexosaminidase and  $\beta$ -D-galactosidase, among allogeneic recipients at day 50 after BMT, isogeneic recipients, and untransplanted controls were not seen in any of the organs examined (data not shown).

## DISCUSSION

After allogeneic BMT for lysosomal storage diseases, the donor-derived blood cells and tissue macrophages, which are enzymatically normal, may serve as a continuous source of enzyme. In the present study, increased enzyme activities are reported in various organs of  $\beta$ -glucuronidase-deficient C3H mice after allogeneic BMT. In mice transplanted with syngeneic bone marrow, no significant increase in  $\beta$ -glucuronidase activity occurred, except for the liver of syngeneic transplanted C57BL in which a prolonged increase in enzyme activity was measured. Differences in hexosaminidase and  $\beta$ -D-galactosidase levels between the untransplanted mice and recipients of syngeneic or allogeneic bone marrow were not seen. These data clearly demonstrate that the observed increases in  $\beta$ -glucuronidase activity in various organs of C3H mice after allogeneic BMT were not due to side effects of the transplantation procedure.

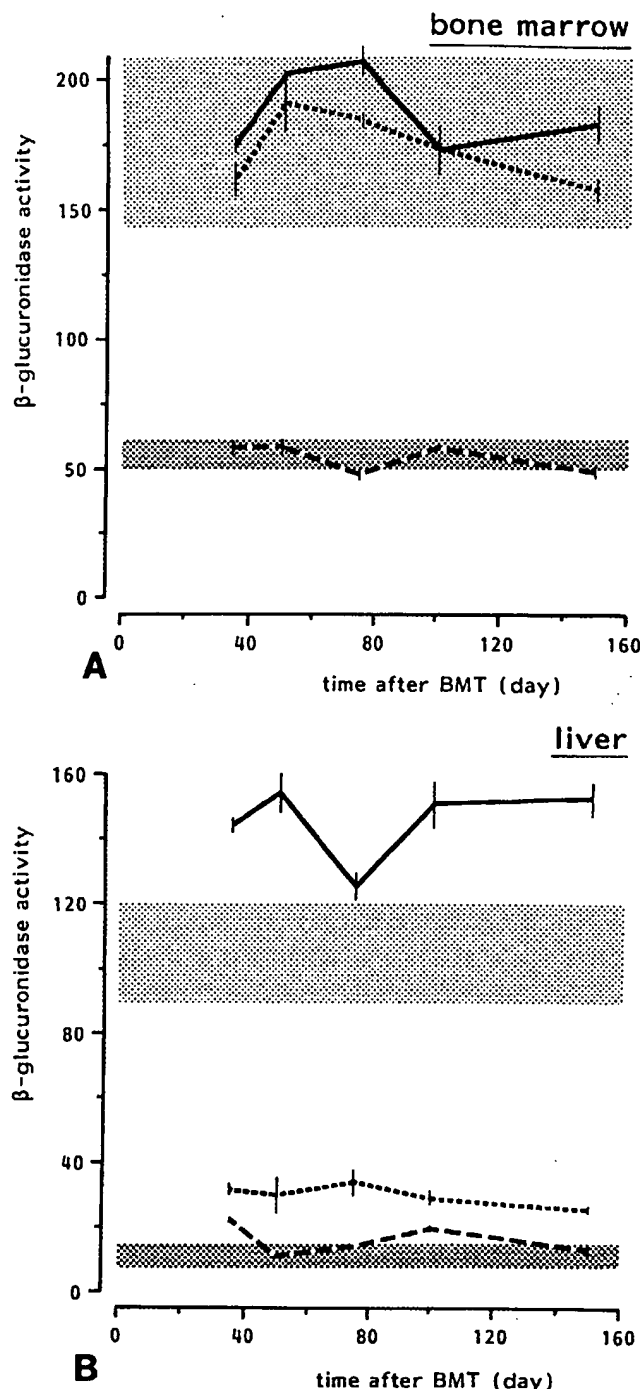


FIGURE 2.  $\beta$ -Glucuronidase activity (nmol/hr/mg prot, mean  $\pm$  SE) in bone marrow (A) and liver (B) at various times after BMT. (—) C57BL to C3H chimera; (---) C57BL recipients of syngeneic BMT; (- - -) C3H recipients of syngeneic BMT. Mean values  $\pm$  2 SD of untransplanted controls are indicated in the shaded areas ([upper] C57BL; [lower] C3H).

It is shown in this study that the increase in enzyme activity after allogeneic BMT varies in the different organs. In the hemopoietic organs (bone marrow and spleen), the enzyme levels increased to those of the donors. This can be explained by the replacement of the hemopoietic cells of these organs by donor-derived cells. The data on the increase in enzyme activity

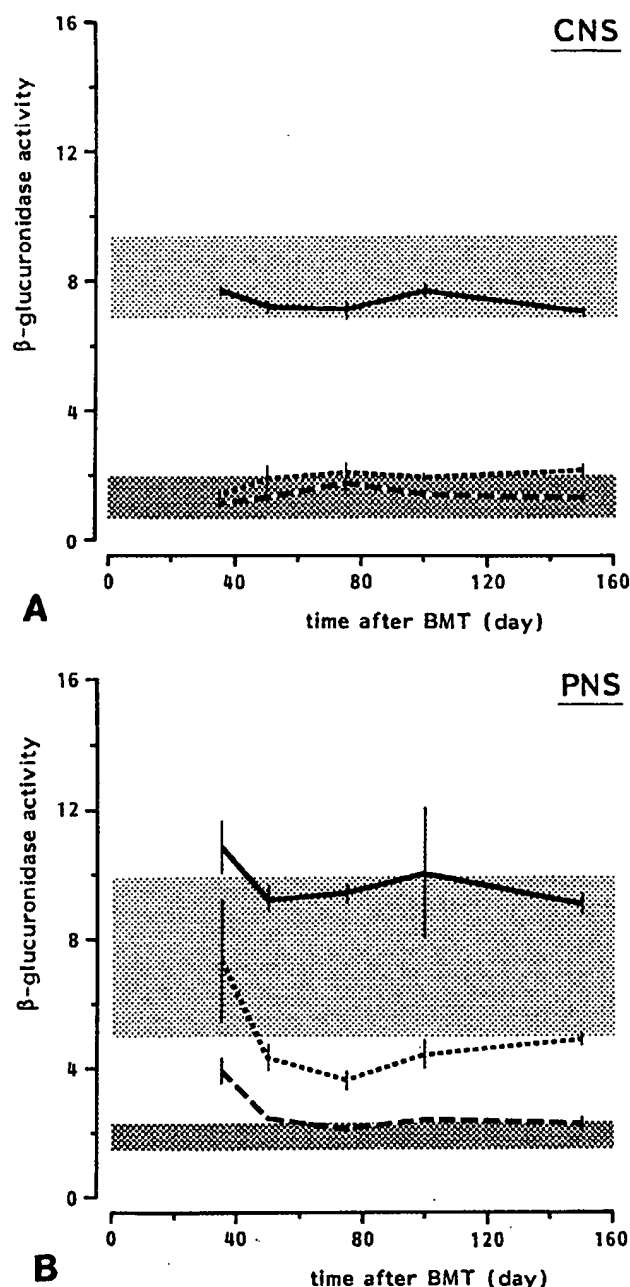


FIGURE 3.  $\beta$ -Glucuronidase activity (nmol/hr/mg prot, mean  $\pm$  SE) in CNS (A) and PNS (B) at various times after BMT. (—) C57BL to C3H chimera; (---) C57BL recipients of syngeneic BMT; (· · ·) C3H recipients of syngeneic BMT. Mean values  $\pm$  2 SD of untransplanted controls are indicated in the shaded areas ([upper] C57BL; [lower] C3H).

in the spleen shown in this study corroborate the clinical decrease in splenomegaly following BMT in patients suffering from certain mucopolysaccharidoses (6, 13). The increased enzyme activity in the plasma is considered to be related to the replacement of the blood cells after BMT and has also been reported in most other studies (6, 15, 16). The increase in  $\beta$ -glucuronidase activity in lung-tissue can be due to replacement of the alveolar macrophages by cells of donor origin (24), or to transfer of  $\beta$ -glucuronidase to the interstitium or to the alveolar and the bronchial cells, or to both. From the present data it

cannot be estimated which mechanism is responsible for the increase in enzyme activity seen in lung tissue.

Also in liver tissue, a significant ( $P < 0.01$ ) increase in enzyme activity was measured after allogeneic BMT. From these data it cannot be concluded whether the increased enzyme activities in liver tissue are only due to replacement of Kupfer cells by donor-type cells (25) or whether they are due to uptake of enzyme by liver parenchymal cells as well.

So far, enzyme activity in the kidney has not been reported. In our study, significantly increased ( $P < 0.01$ )  $\beta$ -glucuronidase levels are observed in the kidney. Whether the increased  $\beta$ -glucuronidase level is only due to replacement of macrophages is questionable, as the presence of macrophages in normal kidneys is controversial. Macrophage-like cells have been reported in normal kidney tissue (26), but recently the presence of these cells in the kidney could not be confirmed (27, 28). Detailed studies of this subject are needed to determine as to whether the effects seen in the present study are due to uptake of enzyme by cells of the kidney or to replacement of macrophages in the kidney after BMT.

The effect of BMT on the  $\beta$ -glucuronidase levels in skeletal muscle is not conclusive. The specific  $\beta$ -glucuronidase levels in skeletal muscle of both C57BL and C3H mice were relatively low as compared with other organs and a large variation in enzyme levels was observed between individual animals of one group. The enzyme activity tended to increase after allogeneic BMT, but this increase was not significant at all time points measured—neither as compared with the untransplanted controls nor as compared with recipients of syngeneic bone marrow. In this respect, it is of interest that another lysosomal enzyme,  $\alpha$ -glucosidase, can be taken up both by deficient Pompe skeletal muscle cells and by normal skeletal muscle cells in vitro (29). It is possible that the enzyme levels in the plasma after BMT are not sufficient to permit uptake by the skeletal muscle cells.

One of the major problems in enzyme replacement therapy for lysosomal storage disease, including BMT, is the neurological complications. Due to the blood-brain barrier, circulating enzyme cannot enter the CNS (30). So far, the presence of donor-derived cells in the CNS has not been reported. One study reported the presence of hemopoietic stem cells in the CNS (31). We have concluded that these "hemopoietic stem cells in the brains" are derived from contamination with bone marrow from the skull bones (32). In the present study, it is shown that after BMT no significant increase in  $\beta$ -glucuronidase level can be observed in the CNS. Although a small difference in enzyme activity was observed, at days 100 and 150 after BMT, between the transplanted mice and the controls, the values were still within the control range. Clinical data (18, 19) and animal model studies (11) indicate that improvement of neurological symptoms does not occur after BMT, but data on enzyme levels have not been reported. In vivo studies with i.v. administration of hexosaminidase (30, 33), showed that uptake of lysosomal enzyme in the brain cells did not occur if the blood-brain barrier was intact, but following artificial opening of the blood-brain barrier, uptake of enzyme was demonstrated. Also, in vitro studies demonstrated that glia cells are able to take up exogenous enzyme in the presence of concanavalin A (24). The present in vivo data do not demonstrate uptake of circulating enzyme in the brain, suggesting that the blood-brain barrier prevents uptake of enzyme in the CNS after BMT.

The mechanism of the increase in  $\beta$ -glucuronidase activity

in peripheral nerves is not well understood. Circulating enzyme can be taken up by Schwann cells. This is suggested to occur in grafted Twitcher nerves, where increased galactosyl-ceramidase levels are reported (35). Yeager et al. (19) have suggested that the improvement of the histological lesions in peripheral nerves in Twitcher mice after BMT may be due to replacement of the macrophages in the demyelinated nerves in Twitcher mice. It is not known whether this also occurs in the histological normal nerves of C3H mice.

If a bone marrow donor is available, BMT may be considered for the treatment of lysosomal storage diseases that are usually fatal and cause severe illness in the affected patients. Our data on leukocytes, spleen, and bone marrow support the view and the reported data (14) that BMT may be beneficial in lysosomal storage diseases in which the cells of the reticuloendothelial system are predominantly affected—e.g., Gaucher's disease (14, 36). However, the present study also shows that in organs that do not contain cells of hemopoietic origin (PNS) or in which the presence of donor-derived cells is dubious (kidney), an increase in enzyme activity does occur after BMT. The increase occurred relatively soon after BMT and appeared to be sustained throughout an observation period of at least 150 days.

Our study shows that significant increase in enzyme activity after BMT cannot be expected in the CNS, suggesting that, for the treatment of lysosomal storage disease which primarily affect the CNS, BMT alone will not be sufficient to ameliorate clinical symptoms. Opening of the blood-brain barrier, in combination with BMT may be effective for these types of lysosomal storage disease, as is indicated by the reports of Neuwelt et al. (30) and Rattazzi (33). They showed that following osmotic modification of the blood-brain barrier in the rat, intraarterially administered human hexosaminidase A and B entered brain cells. The  $\beta$ -glucuronidase deficient C3H/Rij mouse is an appropriate preclinical model in which to evaluate such combined treatment regimens.

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TRANSPLANTATION

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## COMBINED HEART-LUNG TRANSPLANTATION IN THE RAT

### COMPARISON OF THORACIC AND ABDOMINAL OPERATION TECHNIQUES<sup>1</sup>

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Recently, we developed two techniques for the combined transplantation of heart and the left lung into the left hemithorax of rats. One technique, with two vessel anastomoses, comprised the microsurgical repair of aorta, anterior vena cava, and left main bronchus. With the other, single vessel technique, only the aorta and bronchus were anastomosed. In this study, we determined the function and histology of syngeneic and cyclosporine (CsA)-treated allogeneic grafts transplanted with both techniques, and compared the results with those of heterotopic heart-lung grafts transplanted with a previously described technique for transplantation into the rat's abdomen.

The survival rate of rats operated with either of the thoracic transplantation techniques was high (83%). Lungs and hearts of the grafts functioned well for over two months and had normal morphology when the double vessel technique was used. With the single vessel technique, the function of the lungs started to deteriorate from the third postoperative week onward, probably secondary to congestion. The results of thoracic grafts were superior to those of abdominal transplants, where the nonventilated lungs—especially during immunosuppression—were frequently infected. We conclude that these new techniques for thoracic transplan-

tation are most suitable for research of combined heart-lung transplantation.

For the past five years, combined heart-lung transplantation has been used by Stanford University investigators for treatment of patients with end-stage pulmonary vascular disease (1). More than half the transplanted patients surviving the immediate postoperative period have been able to return to a relatively normal life. However, many aspects of heart-lung transplantation still need to be addressed: an adequate preservation method for lungs has yet to be developed (2); noninvasive monitoring of pulmonary rejection is presently virtually impossible (3, 4); and the function of the lungs in some patients has been affected by an obliterative bronchiolitis, the cause of which is unknown (5).

To enable investigation of these troublesome aspects of heart-lung transplantation we recently developed two techniques for combined heart-lung transplantation in rats. Inbred rats have previously been used extensively to study rejection phenomena in isolated orthotopic lung grafts (6-11). With the new techniques, we have been able to study grafts consisting of the heart and left lung transplanted into the left hemithorax after left pneumonectomy. In this way the transplantation provides both an experimental and a control heart and lung in the same animal.

In this study, we compared the function and histology of combined heart-lung grafts transplanted with the two thoracic techniques with those of grafts transplanted with a previously described heterotopic technique (12) into the abdomen. Besides syngeneic grafts, allogeneic transplants with cyclosporine (CsA)\* treatment were used to compare infection susceptibility of the various grafts in immunosuppressed animals.

\* Abbreviations used: CsA, cyclosporine; ECG, electrocardiogram.

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